



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12Q 1/68, C07K 14/435	A1	(11) International Publication Number: WO 00/34526 (43) International Publication Date: 15 June 2000 (15.06.00)
(21) International Application Number: PCT/US99/29405 (22) International Filing Date: 10 December 1999 (10.12.99) (30) Priority Data: 09/210,330 11 December 1998 (11.12.98) US (71) Applicant: CLONTECH LABORATORIES, INC. [US/US]; 1020 East Meadow Drive, Palo Alto, CA 94303 (US). (72) Inventors: LUKYANOY, Sergey Anatolievich; ul. Golubinskaya 13/1-161, Moscow (RU). FRADKOV, Arcady Fedorovich; ul. Dnepropetrovskaya, 35/2-14, Moscow, 113570 (RU). LABAS, Yulii Aleksandrovich; ul. Generala Tyuleneva, 35-416, Moscow, 117465 (RU). MATZ, Mikhail Vladimirovich; ul. Teplyi stan, 7/2-28, Moscow, 117465 (RU). (74) Agent: ADLER, Benjamin, A.; McGregor & Adler, 8011 Candle Ln., Houston, TX 77071 (US).		(81) Designated States: JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: FLUORESCENT PROTEINS FROM NON-BIOLUMINESCENT SPECIES OF CLASS ANTHOZOA, GENES ENCODING SUCH PROTEINS AND USES THEREOF (57) Abstract The present invention is directed to novel fluorescent proteins from non-bioluminescent organisms from the Class Anthozoa. Also disclosed are methods of identifying nucleic acid sequence encoding the fluorescent proteins and further analyzing the proteins.		

Best Available Copy

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

**FLUORESCENT PROTEINS FROM NON-BIOLUMINESCENT SPECIES
OF CLASS ANTHOZOA, GENES ENCODING SUCH PROTEINS AND
USES THEREOF**

5

10

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to the field of molecular biology. More specifically, this invention relates to novel fluorescent proteins, methods of identifying the DNA sequences encoding the proteins and
15 uses thereof.

Description of the Related Art

Fluorescence labeling is a particularly useful tool for
20 marking a protein, cell, or organism of interest. Traditionally, a protein of interest is purified, then covalently conjugated to a fluorophore derivative. For *in vivo* studies, the protein-dye complex is then inserted into cells of interest using micropipetting or a method of reversible permeabilization. The dye attachment and insertion steps,
25 however, make the process laborious and difficult to control. An alternative method of labeling proteins of interest is to concatenate or fuse the gene expressing the protein of interest to a gene expressing a marker, then express the fusion product. Typical markers for this method of protein labeling include β -galactosidase, firefly luciferase

and bacterial luciferase. These markers, however, require exogenous substrates or cofactors and are therefore of limited use for *in vivo* studies.

A marker that does not require an exogenous cofactor or substrate is the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria*, a protein with an excitation maximum at 395 nm, a second excitation peak at 475 nm and an emission maximum at 510 nm. GFP is a 238-amino acid protein, with amino acids 65-67 involved in the formation of the chromophore.

Uses of GFP for the study of gene expression and protein localization are discussed in detail by Chalfie et al. in *Science* 263 (1994), 802-805, and Heim et al. in *Proc. Nat. Acad. Sci.* 91 (1994), 12501-12504. Additionally, Rizzuto et al. in *Curr. Biology* 5 (1995), 635-642, discuss the use of wild-type GFP as a tool for visualizing subcellular organelles in cells, while Kaether and Gerdes in *Febs Letters* 369 (1995), 267-271, report the visualization of protein transport along the secretory pathway using wild-type GFP. The expression of GFP in plant cells is discussed by Hu and Cheng in *Febs Letters* 369 (1995), 331-334, while GFP expression in *Drosophila* embryos is described by Davis et al. in *Dev. Biology* 170 (1995), 726-729.

Crystallographic structures of wild-type GFP and the mutant GFP S65T reveal that the GFP tertiary structure resembles a barrel (Ormö et al., *Science* 273 (1996), 1392-1395; Yang, et al., *Nature Biotechnol* 14 (1996), 1246-1251). The barrel consists of beta sheets in a compact structure, where, in the center, an alpha helix containing the chromophore is shielded by the barrel. The compact structure makes GFP very stable under diverse and/or harsh conditions such as protease treatment, making GFP an extremely useful reporter in

general. However, the stability of GFP makes it sub-optimal for determining short-term or repetitive events.

A great deal of research is being performed to improve the properties of GFP and to produce GFP reagents useful and optimized for a variety of research purposes. New versions of GFP have been developed, such as a "humanized" GFP DNA, the protein product of which has increased synthesis in mammalian cells (Haas, et al., *Current Biology* 6 (1996), 315-324; Yang, et al., *Nucleic Acids Research* 24 (1996), 4592-4593). One such humanized protein is "enhanced green fluorescent protein" (EGFP). Other mutations to GFP have resulted in blue-, cyan- and yellow-green light emitting versions. Despite the great utility of GFP, however, other fluorescent proteins with properties similar to or different from GFP would be useful in the art. Novel fluorescent proteins result in possible new colors, or produce pH-dependent fluorescence. Other benefits of novel fluorescent proteins include fluorescence resonance energy transfer (FRET) possibilities based on new spectra and better suitability for larger excitation.

The prior art is deficient in novel fluorescent proteins wherein the DNA coding sequences are known. The present invention fulfills this long-standing need in the art.

SUMMARY OF THE INVENTION

The present invention is directed to an isolated and purified fluorescent protein selected from the group consisting of amFP486, cFP484, zFP506, zFP538, dsFP483, drFP583, asFP600, dgFP512 and dmFP592.

In one embodiment of the present invention, there is provided a method of identifying a DNA sequence encoding a fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence
5 encodes a peptide having a sequence selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12 and 14. The existence of the nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

In another embodiment of the present invention, there is
10 provided a method of identifying a DNA sequence encoding a fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence hybridizes to a primer selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15 and 16. The existence of the nucleic acid
15 sequence identifies the DNA sequence encoding the fluorescent protein.

In still another embodiment of the present invention, there is provided a method of analyzing a fluorescent protein in a cell, comprising the steps of expressing a nucleic acid sequence encoding a
20 fluorescent protein having an amino acid sequence selected from the group consisting of SEQ ID Nos. 55-63 in the cell; and measuring a fluorescence signal from the protein. This method further comprises a step of sorting the cell according to the signal. Preferably, the cell is sorted by fluorescence activated cell sorting. Still preferably, the
25 nucleic acid sequence comprises a gene of interest encoding a protein of interest fused to the fluorescent protein, wherein the protein of interest is distinct from the fluorescent protein. The detected fluorescence signal indicates the presence of the gene of interest and further the protein of interest in the cell. By identifying an

intracellular location of the fluorescent protein, an intracellular location of the protein of interest is also identified.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the modified strategy of 3'-RACE used to isolate the target fragments. Sequences of the oligonucleotides used are shown in Table 2. Dp1 and Dp2 are the degenerate primers used in the first and second PCR, respectively (see Tables 3 and 4 for the sequences of degenerate primers).

Figure 2A shows multiple alignment of novel fluorescent proteins. The numbering is based on *Aequorea victoria* green fluorescent protein (GFP). Two proteins from *Zoanthus* and four from *Discosoma* are compared between each other: residues identical to the corresponding ones in the first protein of the series are represented by dashes. Introduced gaps are represented by dots. In the sequence of *A. victoria* GFP, the stretches forming beta-sheets are underlined; the residues whose side chains form the interior of the beta-can are shaded (according to Yang et al., *Nature Biotechnol.* 14, 1246-1251 (1996)). **Figure 2B** shows the N-terminal part of cFP484, which has no homology with the other proteins. The putative signal peptide is underlined.

Figure 3 shows the excitation and emission spectrum of the novel fluorescent protein from *Anemonia majano*, amFP486.

Figure 4 shows the excitation and emission spectrum of the novel fluorescent protein from *Clavularia*, cFP484.

Figure 5 shows the excitation and emission spectrum of the novel fluorescent protein from *Zoanthus*, zFP506.

5 **Figure 6** shows the excitation and emission spectrum of the novel fluorescent protein from *Zoanthus*, zFP538.

Figure 7 shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma striata*, dsFP483.

10 **Figure 8** shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma*, drFP583.

Figure 9 shows the excitation and emission spectrum of the novel fluorescent protein from *Anemonia sulcata*, asFP600.

Figure 10 shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma*, dgFP512.

15 **Figure 11** shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma*, dmFP592.

DETAILED DESCRIPTION OF THE INVENTION

20 As used herein, the term "GFP" refers to the basic green fluorescent protein from *Aequorea victoria*, including prior art versions of GFP engineered to provide greater fluorescence or fluoresce in different colors. The sequence of *Aequorea victoria* GFP (SEQ ID No. 54) has been disclosed in Prasher et al., *Gene* 111 (1992), 229-33.

25 As used herein, the term "EGFP" refers to mutant variant of GFP having two amino acid substitutions: F64L and S65T (Heim et al., *Nature* 373 (1995), 663-664). The term "humanized" refers to changes made to the GFP nucleic acid sequence to optimize the codons for

expression of the protein in human cells (Yang et al., *Nucleic Acids Research* 24 (1996), 4592-4593).

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins eds. (1985)); "Transcription and Translation" (B.D. Hames & S.J. Higgins eds. (1984)); "Animal Cell Culture" (R.I. Freshney, ed. (1986)); "Immobilized Cells and Enzymes" (IRL Press, (1986)); B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in either single stranded form or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes.

A DNA "coding sequence" is a DNA sequence which is transcribed and translated into a polypeptide in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3'

(carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and synthetic DNA sequences. A polyadenylation signal and transcription termination
5 sequence may be located 3' to the coding sequence.

As used herein, the term "hybridization" refers to the process of association of two nucleic acid strands to form an antiparallel duplex stabilized by means of hydrogen bonding between residues of the opposite nucleic acid strands.

10 The term "oligonucleotide" refers to a short (under 100 bases in length) nucleic acid molecule.

"DNA regulatory sequences", as used herein, are transcriptional and translational control sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that
15 provide for and/or regulate expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining
20 the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a
25 transcription initiation site, as well as protein binding domains responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Various promoters, including inducible promoters, may be used to drive the various vectors of the present invention.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

A cell has been "transformed" or "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, heterologous DNA includes coding sequence in a construct where portions of genes from two different sources have been brought together so as to produce a fusion protein product. Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

As used herein, the term "reporter gene" refers to a coding sequence attached to heterologous promoter or enhancer elements and whose product may be assayed easily and quantifiably when the construct is introduced into tissues or cells.

5 The amino acids described herein are preferred to be in the "L" isomeric form. The amino acid sequences are given in one-letter code (A: alanine; C: cysteine; D: aspartic acid; E: glutamic acid; F: phenylalanine; G: glycine; H: histidine; I: isoleucine; K: lysine; L: leucine; M: methionine; N: asparagine; P: proline; Q: glutamine; R: arginine; S:
10 serine; T: threonine; V: valine; W: tryptophan; Y: tyrosine; X: any residue). NH₂ refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature, *J Biol. Chem.*, 243 (1969), 3552-
15 59 is used.

The present invention is directed to an isolated and purified fluorescent protein selected from the group consisting of amFP486, cFP484, zFP506, zFP538, dsFP483, drFP583, asFP600, dgFP512 and dmFP592.

20 In one embodiment of the present invention, there is provided a method of identifying a DNA sequence encoding a fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence encodes a peptide having a sequence selected from the group
25 consisting of SEQ ID Nos. 3, 5, 8, 11, 12 and 14. The existence of the nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

In another embodiment of the present invention, there is provided a method of identifying a DNA sequence encoding a

fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence hybridizes to a primer selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15 and 16. The existence of the nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

In still another embodiment of the present invention, there is provided a method of analyzing a fluorescent protein in a cell, comprising the steps of expressing a nucleic acid sequence encoding a fluorescent protein having an amino acid sequence selected from the group consisting of SEQ ID Nos. 55-63 in the cell; and measuring a fluorescence signal from the protein. This method further comprises a step of sorting the cell according to the signal. Preferably, the cell is sorted by fluorescence activated cell sorting. Still preferably, the nucleic acid sequence comprises a gene of interest encoding a protein of interest fused to the fluorescent protein, wherein the protein of interest is distinct from the fluorescent protein. The detected fluorescence signal indicates the presence of the gene of interest and further the protein of interest in the cell. By identifying an intracellular location of the fluorescent protein, an intracellular location of the protein of interest is also identified.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1Biological Material

- 5 Novel fluorescent proteins were identified from several genera of Anthozoa which do not exhibit any bioluminescence but have fluorescent color as observed under usual white light or ultraviolet light. Six species were chosen (see Table 1).

10

TABLE 1Anthozoa Species Used in This Study

Species	Area of Origination	Fluorescent Color
Anemonia majano	Western Pacific	bright green tentacle tips
Clavularia sp.	Western Pacific	bright green tentacles and oral disk
Zoanthus sp.	Western Pacific	green-yellow tentacles and oral disk
Discosoma sp. "red"	Western Pacific	orange-red spots oral disk
Discosoma striata	Western Pacific	blue-green stripes on oral disk
Discosoma sp. "magenta"	Western Pacific	faintly purple oral disk
Discosoma sp.	Western Pacific	green spots on oral disk

"green"		
Anemonia sulcata	Mediterranean	purple tentacle tips

EXAMPLE 2

cDNA Preparation

Total RNA was isolated from the species of interest according to the protocol of Chomczynski and Sacchi (Chomczynski P., et al., *Anal. Biochem.* 162 (1987), 156-159). First-strand cDNA was synthesized starting with 1-3 µg of total RNA using SMART PCR cDNA synthesis kit (CLONTECH) according to the provided protocol with the only alteration being that the "cDNA synthesis primer" provided in the kit was replaced by the primer TN3 (5'- CGCAGTCGACCG(T)₁₃, SEQ ID No. 1) (Table 2). Amplified cDNA samples were then prepared as described in the protocol provided except the two primers used for PCR were the TS primer (5'-AAGCAGTGGTATCAACGCAGAGT, SEQ ID No. 2) (Table 2) and the TN3 primer (Table 2), both in 0.1 µM concentration. Twenty to twenty-five PCR cycles were performed to amplify a cDNA sample. The amplified cDNA was diluted 20-fold in water and 1 µl of this dilution was used in subsequent procedures.

TABLE 2

Oligos Used in cDNA Synthesis and RACE

5 TN3: 5'-CGCAGTCGACCG(T)₁₃
(SEQ ID No. 1)

T7-TN3: 5'-GTAATACGACTCACTATAGGGCCGCGAGTCGACCG(T)₁₃
(SEQ ID No. 17)

10

TS-primer: 5'-AAGCAGTGGTATCAACGCAGAGT
(SEQ ID No. 2)

T7-TS:
15 5'-GTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
(SEQ ID No. 18)

T7: 5'-GTAATACGACTCACTATAGGGC
(SEQ ID No. 19)

20

TS-oligo 5'-AAGCAGTGGTATCAACGCAGAGTACGCrGrGrG
(SEQ ID No. 53)

25

EXAMPLE 3Oligo Design

To isolate fragments of novel fluorescent protein cDNAs,
5 PCR using degenerate primers was performed. Degenerate primers
were designed to match the sequence of the mRNAs in regions that
were predicted to be the most invariant in the family of fluorescent
proteins. Four such stretches were chosen (Table 3) and variants of
degenerate primers were designed. All such primers were directed to
10 the 3'-end of mRNA. All oligos were gel-purified before use. Table 2
shows the oligos used in cDNA synthesis and RACE.

TABLE 3

Key Amino Acid Stretches and Corresponding Degenerate Primers Used for Isolation of Fluorescent Proteins

5

Stretch Position according to A. victoria GFP (7)	Amino Acid Sequence of the Key Stretch	Degenerated Primer Name and Sequence
20-25	GXVNGH (SEQ ID No. 3)	NGH: 5'- GA(C,T) GGC TGC GT(A,T,G,C) AA(T,C) GG(A,T,G) CA (SEQ ID No. 4)
31-35	GEGEG (SEQ ID No. 5) GEGNG (SEQ ID No. 8)	GEGa: 5'- GTT ACA GGT GA(A,G) GG(A,C) GA(A,G) GG (SEQ ID No. 6) GEGb: 5'- GTT ACA GGT GA(A,G) GG(T,G) GA(A,G) GG (SEQ ID No. 7) GNGa: 5'- GTT ACA GGT GA(A,G) GG(A,C) AA(C,T) GG (SEQ ID No. 9) GNGb: 5'- GTT ACA GGT GA(A,G) GG(T,G) AA(C,T) GG (SEQ ID No. 10)
127-131	GMNFP (SEQ ID No. 11) GVNFP (SEQ ID No. 12)	NFP: 5' TTC CA(C,T) GGT (G,A)TG AA(C,T) TT(C,T) CC (SEQ ID NO. 13)
134-137	GPVM (SEQ ID No. 14)	PVMa: 5' CCT GCC (G,A)A(C,T) GGT CC(A,T,G,C) GT(A,C) ATG (SEQ ID NO. 15) PVMb: 5' CCT GCC (G,A)A(C,T) GGT CC(A,T,G,C) GT(G,T) ATG (SEQ ID NO. 16)

EXAMPLE 4Isolation of 3'-cDNA Fragments of nEPs

The modified strategy of 3'-RACE was used to isolate the
5 target fragments (see Figure 1). The RACE strategy involved two
consecutive PCR steps. The first PCR step involved a first degenerate
primer (Table 4) and the T7-TN3 primer (SEQ ID No. 17) which has a 3'
portion identical to the TN3 primer used for cDNA synthesis (for
sequence of T7-TN3, Table 2). The reason for substituting the longer
10 T7-TN3 primer in this PCR step was that background amplification
which occurred when using the shorter TN3 primer was suppressed
effectively, particularly when the T7-TN3 primer was used at a low
concentration (0.1 μ M) (Frohman et al., (1998) *PNAS USA*, 85, 8998-
9002). The second PCR step involved the TN3 primer (SEQ ID No. 1,
15 Table 2) and a second degenerate primer (Table 4).

TABLE 4

Combinations of Degenerate Primers for First and Second PCR Resulting in Specific Amplification of 3'-Fragments of nFP cDNA

Species	First Degenerate Primer	Second Degenerate Primer
Anemonia majano	NGH (SEQ ID No. 4)	GNGb (SEQ ID No. 10)
Clavularia sp.	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6)
Zoanthus sp.	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6)
Discosoma sp. "red"	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6), NFP (SEQ ID No. 13) or PVMb (SEQ ID No. 16)
Discosoma striata	NGH (SEQ ID No. 4)	NFP (SEQ ID No. 13)
Anemonia sulcata	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6) or NFP (SEQ ID No. 13)

5

The first PCR reaction was performed as follows: 1 µl of 20-fold
 10 dilution of the amplified cDNA sample was added into the reaction
 mixture containing 1X Advantage KlenTaq Polymerase Mix with
 provided buffer (CLONTECH), 200 µM dNTPs, 0.3 µM of first degenerate

primer (Table 4) and 0.1 μ M of T7-TN3 (SEQ ID No. 17) primer in a total volume of 20 μ l. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 1 cycle for 95°C, 10 sec.; 55°C, 1 min.; 72°C, 40 sec; 24 cycles for 95°C, 10 sec.; 62°C, 30 sec.; 72°C, 40 sec. The reaction was then diluted 20-fold in water and 1 μ l of this dilution was added to a second PCR reaction, which contained 1X Advantage KlenTaq Polymerase Mix with the buffer provided by the manufacturer (CLONTECH), 200 μ M dNTPs, 0.3 μ M of the second degenerate primer (Table 4) and 0.1 μ M of TN3 primer. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 1 cycle for 95°C, 10 sec.; 55°C (for GEG/GNG or PVM) or 52°C (for NFP), 1 min.; 72°C, 40 sec; 13 cycles for 95°C, 10sec.; 62°C (for GEG/GNG or PVM) or 58°C (for NFP), 30 sec.; 72°C, 40 sec. The product of PCR was cloned into PCR-Script vector (Stratagene) according to the manufacturer's protocol.

Different combinations of degenerate primers were tried in the first and second PCR reactions on the DNA from each species until a combination of primers was found that resulted in specific amplification--meaning that a pronounced band of expected size (about 650-800 bp for NGH and GEG/GNG and 350-500 bp for NFP and PVM--sometimes accompanied by a few minor bands) was detected on agarose gel after two PCR reactions. The primer combinations of choice for different species of the Class Anthozoa are listed in Table 4. Some other primer combinations also resulted in amplification of fragments of correct size, but the sequence of these fragments showed no homology to the other fluorescent proteins identified or to *Aequorea victoria* GFP.

EXAMPLE 5

Obtaining Full-Length cDNA Copies

Upon sequencing the obtained 3'-fragments of novel
5 fluorescent protein cDNAs, two nested 5'-directed primers were
synthesized for cDNA (Table 5), and the 5' ends of the cDNAs were
then amplified using two consecutive PCRs. In the next PCR reaction,
the novel approach of "step-out PCR" was used to suppress background
amplification. The step-out reaction mixture contained 1x Advantage
10 KlenTaq Polymerase Mix using buffer provided by the manufacturer
(CLONTECH), 200 μ M dNTPs, 0.2 μ M of the first gene-specific primer
(see Table 5), 0.02 μ M of the T7-TS primer (SEQ ID No. 18), 0.1 μ M of
T7 primer (SEQ ID No. 19) and 1 μ l of the 20-fold dilution of the
amplified cDNA sample in a total volume of 20 μ l. The cycling profile
15 was (Hybaid OmniGene Thermocycler, tube control mode): 23-27
cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of
amplification was diluted 50-fold in water and one μ l of this dilution
was added to the second (nested) PCR. The reaction contained 1X
Advantage KlenTaq Polymerase Mix with provided buffer (CLONTECH),
20 200 μ M dNTPs, 0.2 μ M of the second gene-specific primer and 0.1 μ M
of TS primer (SEQ ID No. 2) in a total volume of 20 μ l. The cycling
profile was (Hybaid OmniGene Thermocycler, tube control mode): 12
cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of
amplification was then cloned into pAtlas vector (CLONTECH) according
25 to the manufacturer's protocol.

TABLE 5Gene-Specific Primers Used for 5'-RACE

Species	First Primer	Second (Nested) Primer
Anemonia majano	5'-GAAATAGTCAGGCATACTGGT (SEQ ID No. 20)	5'-GTCAGGCATAC TGGTAGGAT (SEQ ID No. 21)
Clavularia sp.	5'-CTTGAAATAGTCTGCTATATC (SEQ ID No. 22)	5'-TCTGCTATATC GTCTGGGT (SEQ ID No. 23)
Zoanthus sp.	5'- GTTCTTGAAATAGTCTACTATGT (SEQ ID No. 24)	5'-GTCTACTATGTCTT GAGGAT (SEQ ID No. 25)
Discosoma sp. "red"	5'-CAAGCAAATGGCAAAGGTC (SEQ ID No. 26)	5'-CGGTATTGTGGCC TTCGTA (SEQ ID No. 27)
Discosoma striata	5'-TTGTCTTCTTCTGCACAAC (SEQ ID No. 28)	5'-CTGCACAACGG GTCCAT (SEQ ID No. 29)
Anemonia sulcata	5'-CCTCTATCTTCATTTCCTGC (SEQ ID No. 30)	5'-TATCTTCATTTCCT GCGTAC (SEQ ID No. 31)
Discosoma sp. "magenta"	5'-TTCAGCACCCCATCACGAG (SEQ ID No. 32)	5'-ACGCTCAGAGCTG GGTTCC (SEQ ID No. 33)
Discosoma sp. "green"	5'-CCCTCAGCAATCCATCACGTTC (SEQ ID No. 34)	5'-ATTATCTCAGTGGA TGGTTC (SEQ ID No. 35)

EXAMPLE 6

Expression of nFPs in *E. coli*

5 To prepare a DNA construct for novel fluorescent protein expression, two primers were synthesized for each cDNA: a 5'-directed "downstream" primer with the annealing site located in the 3'-UTR of the cDNA and a 3'-directed "upstream" primer corresponding to the site of translation start site (not including the first ATG codon) (Table 10 6). Both primers had 5'-heels coding for a site for a restriction endonuclease; in addition, the upstream primer was designed so as to allow the cloning of the PCR product into the pQE30 vector (Qiagen) in such a way that resulted in the fusion of reading frames of the vector-encoded 6xHis-tag and nFP. The PCR was performed as follows: 1 µl of 15 the 20-fold dilution of the amplified cDNA sample was added to a mixture containing 1x Advantage KlenTaq Polymerase Mix with buffer provided by the manufacturer (CLONTECH), 200 µM dNTPs, 0.2 µM of upstream primer and 0.2 µM of downstream primer, in a final total volume of 20 µl. The cycling profile was (Hybaid OmniGene 20 Thermocycler, tube control mode): 23-27 cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of this amplification step was purified by phenol-chlorophorm extraction and ethanol precipitation and then cloned into pQE30 vector using restriction endonucleases corresponding to the primers' sequence according to standard 25 protocols.

All plasmids were amplified in XL-1 blue *E. coli* and purified by plasmid DNA miniprep kits (CLONTECH). The recombinant clones were selected by colony color, and grown in 3 ml of LB medium (supplemented with 100 µg/ml of ampicillin) at 37°C overnight. 100 µl

of the overnight culture was transferred into 200 ml of fresh LB medium containing 100 μ g/ml of ampicillin and grown at 37°C, 200 rpm up to OD₆₀₀ 0.6-0.7. 1 mM IPTG was then added to the culture and incubation was allowed to proceed at 37°C for another 16 hours. The
5 cells were harvested and recombinant protein, which incorporated 6x His tags on the N-terminus, was purified using TALON™ metal-affinity resin according to the manufacturer's protocol (CLONTECH).

TABLE 6

Primers Used to Obtain Full Coding Region of nFPs for Cloning into Expression Construct

Species	Upstream Primer	Downstream Primer
Anemonia majano	5' -acatggatccgctcttttcaaaca agttatc (SEQ ID No. 36) BamHI	5'-tagtactcgagcttattcgta tttcagtgaatc (SEQ ID No. 37) XhoI
Clavularia sp.	L: 5'-acatggatccaacattttttga gaaacg (SEQ ID No. 38) BamHI S: 5'-acatggatccaaagctctaacc accatg (SEQ ID No. 39) BamHI	5'-tagtactcgagcaacacaa accctcagacaa (SEQ ID No. 40) XhoI
Zoanthus sp.	5'- acatggatccgctcagtcacaaag cacggt (SEQ ID No. 41) BamHI	5'-tagtactcgaggttggaactacat tcttatca (SEQ ID No. 42) XhoI
Discosoma sp. "red"	5'- acatggatccaggtcttccaagaat gttatc (SEQ ID No. 43) BamHI	5'-tagtactcgaggagccaagttc agcctta (SEQ ID No. 44) XhoI
Discosoma striata	5'- acatggatccagttggtccaagagtgtg (SEQ ID No. 45) BamHI	5'-tagcgagctctatcatgcctc gtcacct (SEQ ID No. 46) SacI
Anemonia sulcata	5'- acatggatccgcttccttttaagaagact (SEQ ID No. 47) BamHI	5'-tagtactcgagtccttgggagc ggcttg (SEQ ID No. 48) XhoI
Discosoma sp. "magenta"	5'- acatggatccagttgtccaagaatgtgat (SEQ ID No. 49) BamHI	5'-tagtactcgaggccattacg ctaate (SEQ ID No. 50) XhoI
Discosoma sp. "green"	5'-acatggatccagtcacttaagaagaagaatg (SEQ ID No. 51)	5'-tagtactcgagattcggtttaat gccttg (SEQ ID No. 52)

EXAMPLE 7Novel Fluorescent Proteins and cDNAs Encoding the Proteins

Seven cDNA full-length cDNAs encoding fluorescent
5 proteins were obtained (SEQ ID Nos. 45-51), and seven novel
fluorescent proteins were produced (SEQ ID Nos. 53-59). The spectral
properties of the isolated novel fluorescent proteins are shown in Table
7, and the emission and excitation spectra for the novel proteins are
shown in Figures 3-11.

10

TABLE 7Spectral Properties of the Isolated NFPs.

Species	NFP Name	Abs. Max. n m	Emission Maximum n m	Maximum Extinction Coeff.	Relative Quantum Yield*	Relative Brightness **
Anemonia majano	amFP486	458	486	40,000	0.3	0.43
Clavularia sp.	cFP484	456	484	35,300	0.6	0.77
Zoanthus sp.	zFP506	496	506	35,600	0.79	1.02
Zoanthus sp.	zFP538	528	538	20,200	0.52	0.38
Discosoma sp. "red"	drFP583	558	583	22,500	0.29	0.24
Discosoma striata	dsFP483	443	483	23,900	0.57	0.50
Anemonia sulcata	asFP600	572	596	56,200	<0.001	-
Discosoma sp "green"	dgFP512	502	512	20,360	0.3	0.21
Discosoma sp. "magenta"	dmFP592	573	593	21,800	0.11	0.09

5 *relative quantum yield was determined as compared to the quantum yield of *A. victoria* GFP.

**relative brightness is extinction coefficient multiplied by quantum yield divided by the same value for *A. victoria* GFP.

Multiple alignment of fluorescent proteins is shown in Figure 2A. The numbering is based on *Aequorea victoria* green fluorescent protein (GFP, SEQ ID No. 54). The amino acid sequences of the novel fluorescent proteins are labeled as SEQ ID Nos. 55-63. Two proteins from *Zoanthus* and four from *Discosoma* are compared between each other: residues identical to the corresponding ones in the first protein of the series are represented by dashes. Introduced gaps are represented by dots. In the sequence of *A. victoria* GFP, the stretches forming β -sheets are underlined; the residues whose side chains form the interior of the β -can are shaded. Figure 2B shows the N-terminal part of cFP484, which has no homology with the other proteins. The putative signal peptide is underlined.

The following references were cited herein.

1. Ormo et al., (1996) Science 273: 1392-1395.
2. Yang, F., et al., (1996) Nature Biotech 14: 1246-1251.
3. Cormack, et al., (1996) Gene 173, 33-38.
4. Haas, et al., (1996) Current Biology 6, 315-324.
5. Yang, et al., (1996) Nucleic Acids Research 24, 4592-4593.
6. Ghoda, et al., (1990) J. Biol. Chem. 265: 11823-11826.
7. Prasher D.C. et al. (1992) Gene 111:229-33.
8. Kain et al. (1995) Biotechniques 19(4):650-55.
9. Chomczynski P., et al., (1987) Anal. Biochem. 162, 156-159.
10. Frohman et al., (1998) PNAS USA, 85, 8998-9002.

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

1. A method of identifying a DNA sequence encoding a fluorescent protein, comprising the step of:

5 screening for an existence of a nucleic acid sequence in a sample, wherein said nucleic acid sequence encodes a peptide having a sequence selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12 and 14, and wherein the existence of said nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

10 2. A method of identifying a DNA sequence encoding a fluorescent protein, comprising the step of:

15 screening for an existence of a nucleic acid sequence in a sample, wherein said nucleic acid sequence hybridizes to a primer selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15 and 16, and wherein the existence of said nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

20 3. A method of analyzing a fluorescent protein in a cell, comprising the steps of:

a) expressing a nucleic acid sequence encoding a fluorescent protein in said cell, wherein said protein having an amino acid sequence selected from the group consisting of SEQ ID Nos. 55-63; and

25 b) measuring a fluorescence signal from said protein.

4. The method of claim 3, further comprising the step of:

sorting said cell according to said signal.

5. The method of claim 4, wherein said step of sorting comprises sorting said cell by fluorescence activated cell sorting.

5 6. The method of claim 3, wherein said nucleic acid sequence comprises a gene of interest encoding a protein of interest fused to said fluorescent protein, wherein said protein of interest is distinct from said fluorescent protein.

10 7. The method of claim 6, wherein the fluorescence signal indicates a presence of said gene of interest in said cell.

8. The method of claim 7, wherein said cell further comprises a protein of interest fused to said fluorescent protein.

15 9. The method of claim 8, further comprising the step of:

identifying an intracellular location of said fluorescent protein, thereby identifying an intracellular location of said protein of
20 interest.

10. An isolated and purified fluorescent protein selected from the group consisting of amFP486, cFP484, zFP506, zFP538, dsFP483, drFP583, asFP600, dgFP512 and dmFP592.

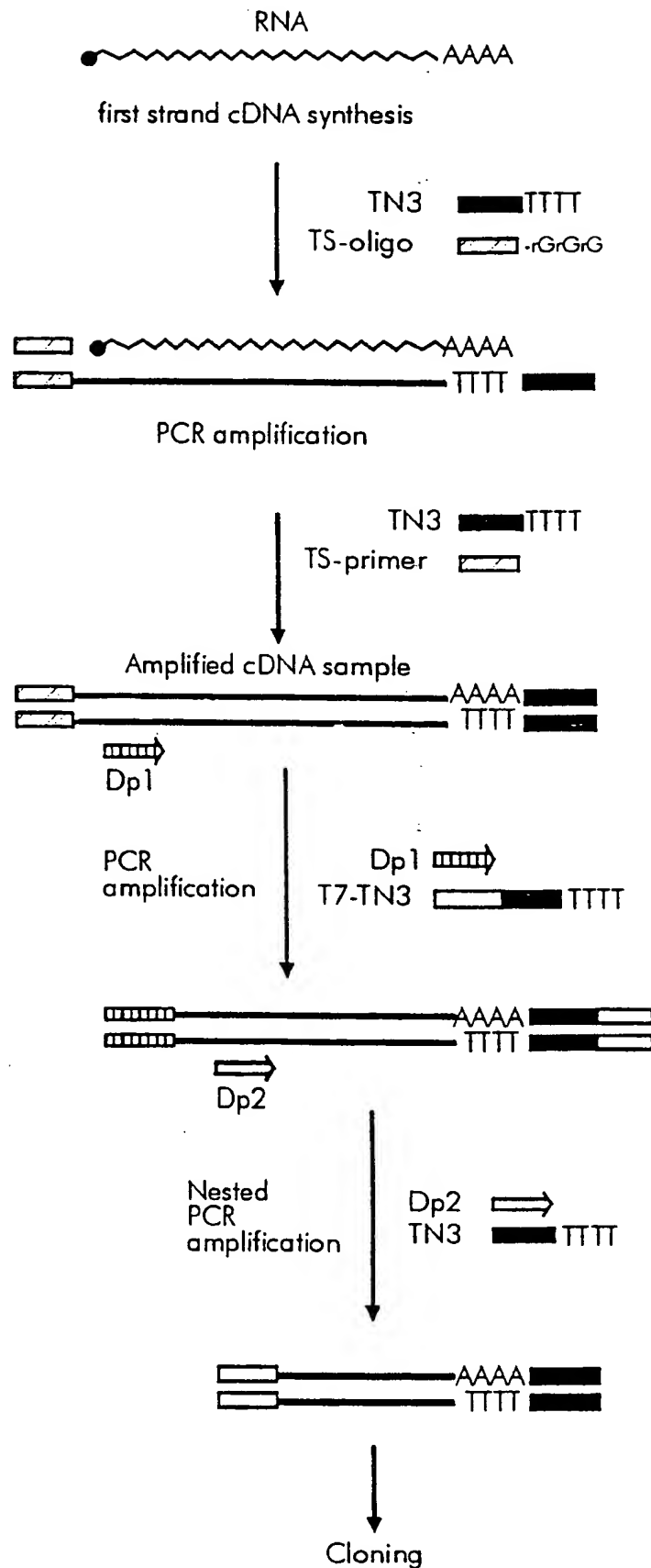


FIG. 1
1/11

SUBSTITUTE SHEET (RULE 26)

10	20	30	40	50	SEQ ID#
MSKGEELFTG.	VVPILVELDGDVNGHKFSVSGEGEGDATY	GKLTILKFICTT.	GKLPVP..	W GFP	54
MAQSKHGLTK.	EMTMKYRMEGCV	DGHKFVITGEGIGY	PFKKGQAINLCVV..	EGGPLPFAE	zFP506
--H-----KE.	-----H-----N-----	-----T-----I.	-----S-	zFP538	57
MSWSKSVIKE.	EMLIDLHLEGT	FNGHYFEIKGKGKGP	NEGTNTVTLEVT..	KGGPLPFGW	dsFP483
...M-AL--	Y-K-N-TM--	VV--LP-K-R-D-----	YQ-SQEL--T-V..	-----SY	dgFP512
-RS--N----	F-RFKVRM--	V---E---E-E-E-R-Y--	H---K-K--	-----A-	drFP583
M-C--N----	F-RFKVRM--	V---E-----E-E-R-Y--	HCS-K-M--	-----AF	dmFP592
...MASFLKK.	TMPFKTTIEGT	VNGHYFKCTGKGEGNP	FEGTQEMKIEVI..	EGGPLPFAE	asFP600
MALSNKFIGD.	DMKMTYIIMDGC	VNGHYFTVKGEGNGKPY	EGTQTSTFKVTMANGG	PLAFSF	amFP486
«KALTMTMGVIK	PDMKIKLMEGNVNGHA	FVIEGEGEGKPYDGTHT	LNLEVKMAEGAPLPFSY	cFP484	56
60	70	80	90	100	110
PTLVTTFSYGVQCFS	SRYPDHMKQHDFK	SAM..	PEGYVQERTIFFKDDG	NYKTRA	EVKFE
DILSAAFNYGNRV	FETEYPQDIV..	DYFKNSC...	PAGYTWDRSFLFEDG	AVCICNADITVS	VEEN
-----G-K--D-I-	-----	-----G-----	V-----	K--	
HILCPQFQYGNKA	FAFVHPDDIP..	DYLKLSF...	PEGYTWERSMHFEDG	GLCCITNDISLTGN..	
D--TTM-----	R--NY-E---	IF-QTCSPNG--	S-Q-T-TY---	V-TA-SN--	VV-D..
D--S-----	S-VY-K--A---	K-----	FK--V-N----	VVTV-Q-S--	QDG..
D--S-----	S-VY-K--A---	K-----	FK--V-N----	VVTVSQ-S--	KDG..
HILSTSCMYGSKT	FIKYVSGIP..	DYFKQSF...	PEGFTWERTTTTYEDG	GFLTAHQDTS	LDGD..
DILSTVFKYGNRC	FAYPTSMF..	DYFKQAF...	PDGMSYERTFTYEDG	GVTASWEISL	KGN..
DILSNAFQYGNRAL	TKYPDDIA..	DYFKQSF...	PEGYSWERTMTFEDK	GIVKVKSDIS	MEED..
120	130	140	150	160	170
TLVNRIELKGIDF	KEDGNILGHKLEYN	YNNSHNVYIMADKQ	KNGIKVNFKIRHN	IEDG	SVQL
CMYHESKFYGVNF	PADGPVM.	KKMTDNWEP	SCEKII	PVPKQGI	LKGDVSMYLL
-I--K-I-N-M-----	-----T--A-----	M-----	Y-		
CENYDIKFTGLNF	PNGPVV.QKKT	TGWEPSTERLYP..	RDGVLIGDIHHA	LTVEGGGHYV	
T-----H-M-A---	LD--MM.--R-MK-----	IMFE	---L-R-D-AMS-LLK-----	R	
--I-KV--I-V---	SD--M.-----M--A-----	-----K-E--K--KLKD----	L		
--I-EV--I-V---	SD--M.-RR-R-----S-----	-----K-----M--RL-----	L		
CLVYKVKILGNNF	PADGPVM.QNKAGR	WEPATEIVYE..	VGVLRGQSLMALK	CPGGRHLT	
CFEHKSTFHGVNF	PADGPVM.AKKT	TGWDPSEKMTV..	CDGILKGDVTAFL	MLQGGSNYR	
SFIYEIRFDGMNF	PNGPVV.QKKT	LKWEPESTEIMYV..	RDGVLVGDISH	SLLEGGGHYR	
180	190	200	210	220	230
ADHYQONTPIGDG.	PVLLPDNHYLSTQ	SALSKDPNEKR	DHMLLEFVTAAG	ITHGMDELYK	
CQFDTVYKAKSV..	PRKMPDWHFIQ	KLTRSDAKNQK	WHLTEHAIASGS	ALP	
-----S---	E-----L-----	Q-----	FP---	A	
CDIKVYQAKK...	PVKMPGYHYVD	TKLVIRSNDKEFM.	KVEEHEIAVARH	HPLOSQ	
--FE-I-KPN-	V----	D--F--HYIE-T-QQ	NYN V--LT-V-E--	YSS-EKIGKSKA	
VEF-SI-M---QL---	Y--S--D-T-HNEDYT.	I--QY-RTEG---	LFL	
VEF-SI-MV--	PS-QL---	Y--S--DMT-HNEDYT	V--QY-KTQ-----	FIKPLQ	
CHLHTTYRSKKP	ASALKMPGFH	FEDHRIEIMEEVEK	GK.CYKQYEA	AVGRYCDAA	PSKLGHN
CQFHTSYKTKK...	PVTMPNHN	VEHRIARTDL	DKGGN.SVOLTE	HAVA	HITSVF
CDFKSIYKAKK...	VVKLPDYH	FVDHRIEILN	HDKDYN.KVTLY	ENAVARYS	LLPSQA

FIG. 2A

»
MKCKFVFCLSLVLAITNANIFLRNEADLEEKTLRIP

FIG. 2B

2/11

SUBSTITUTE SHEET (RULE 26)

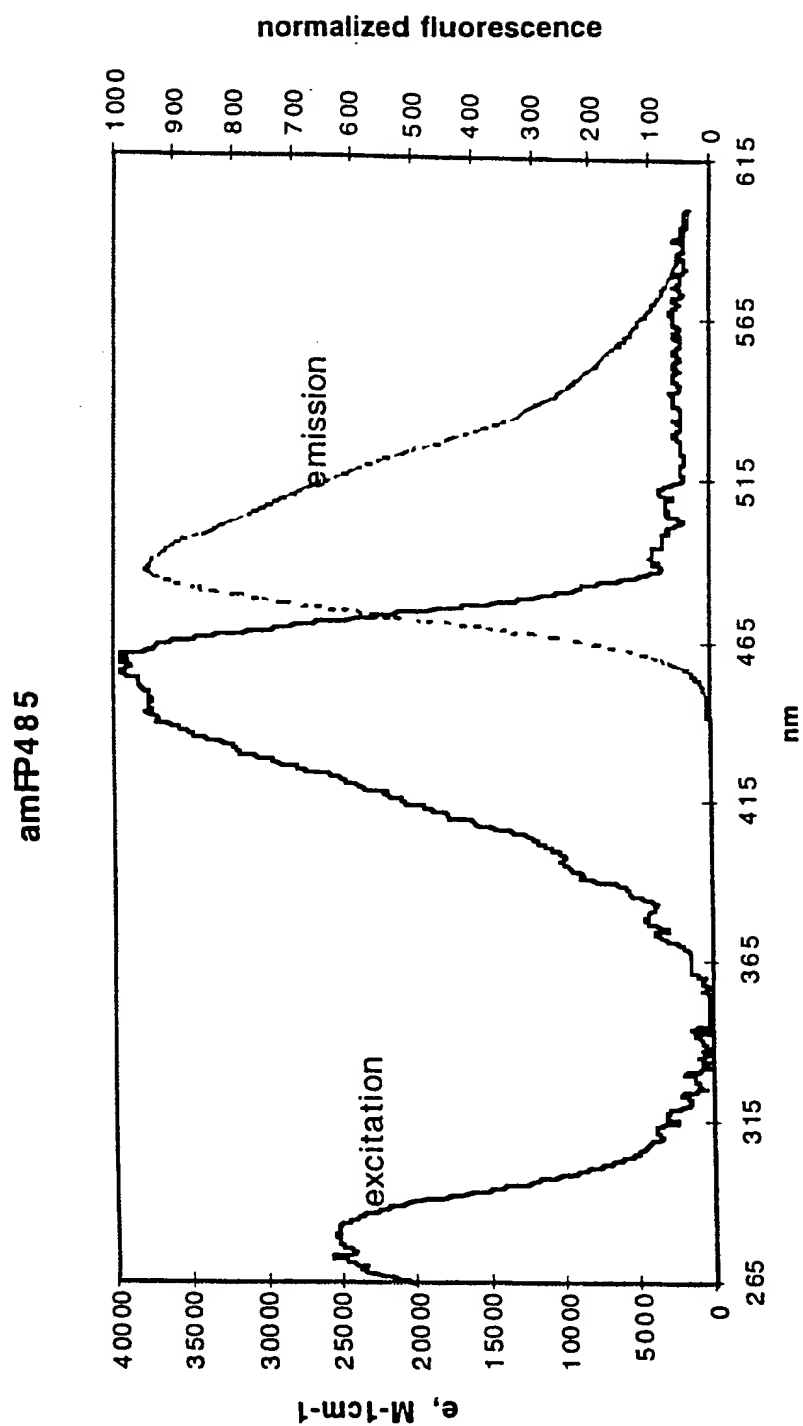


FIG. 3

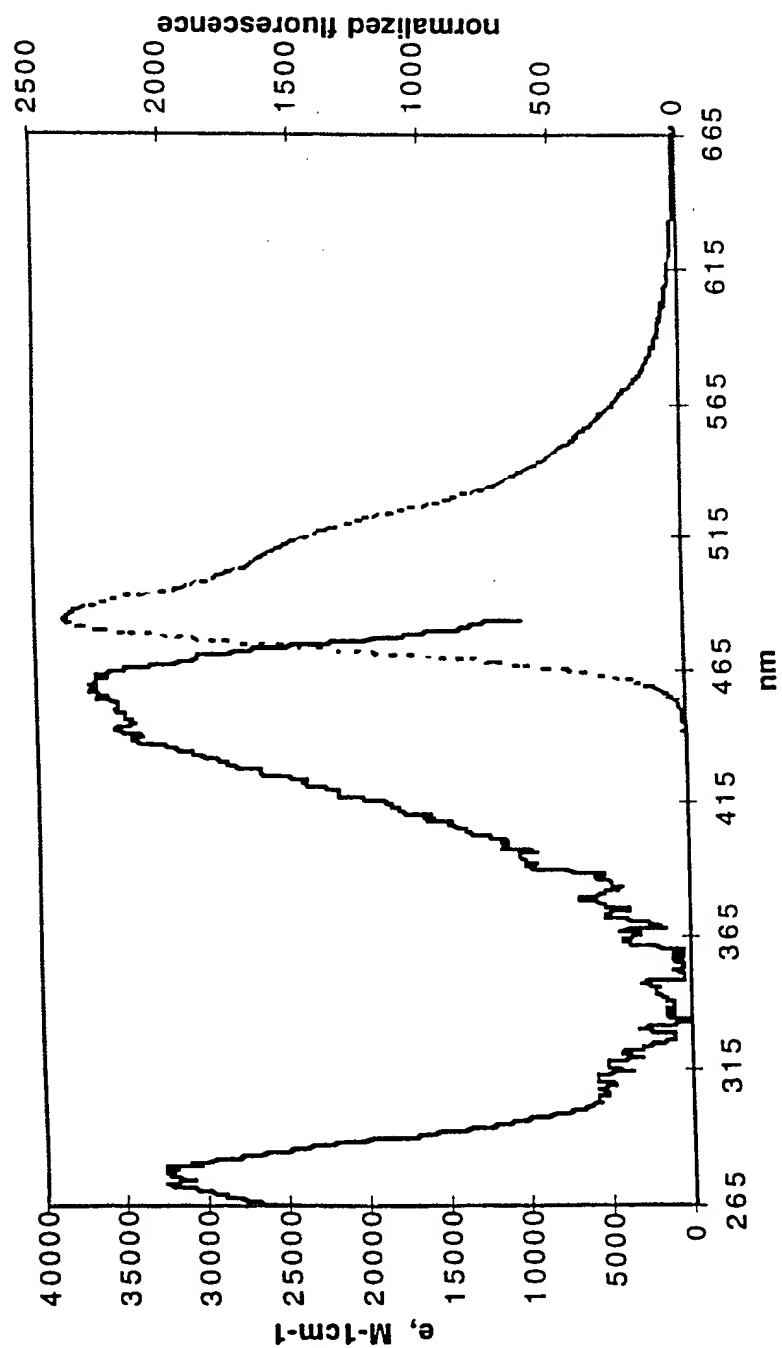


FIG. 4

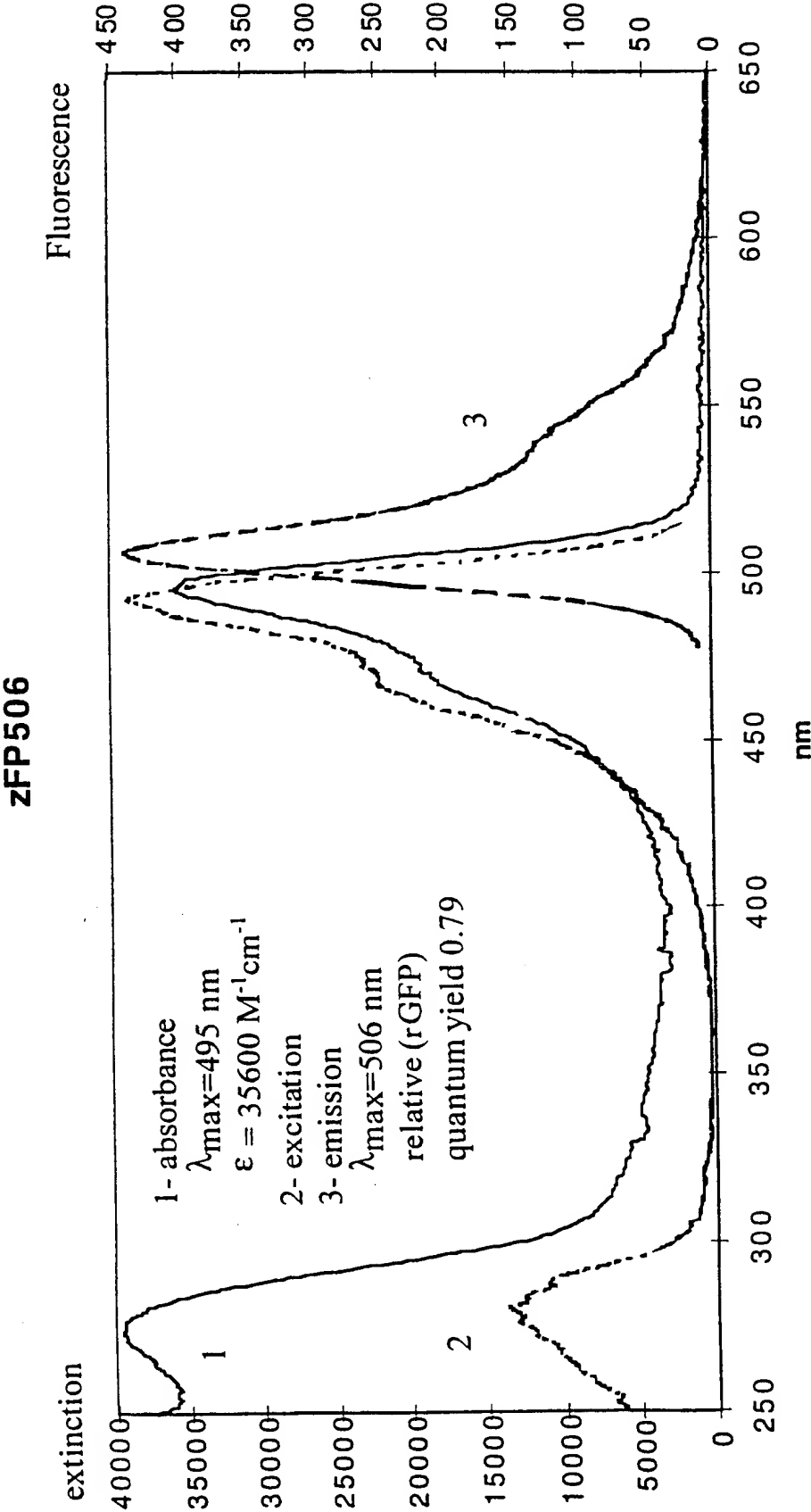


FIG. 5

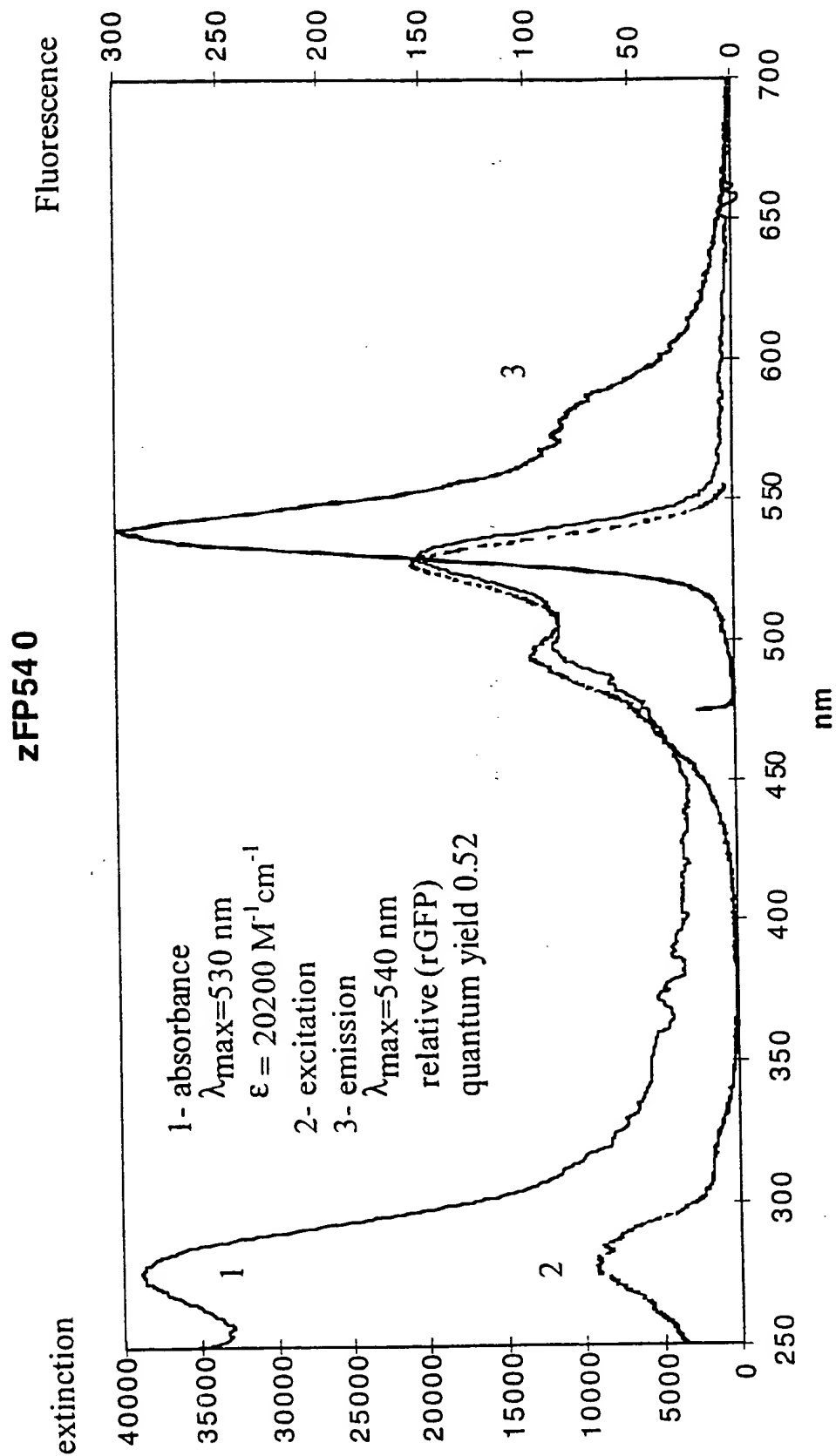
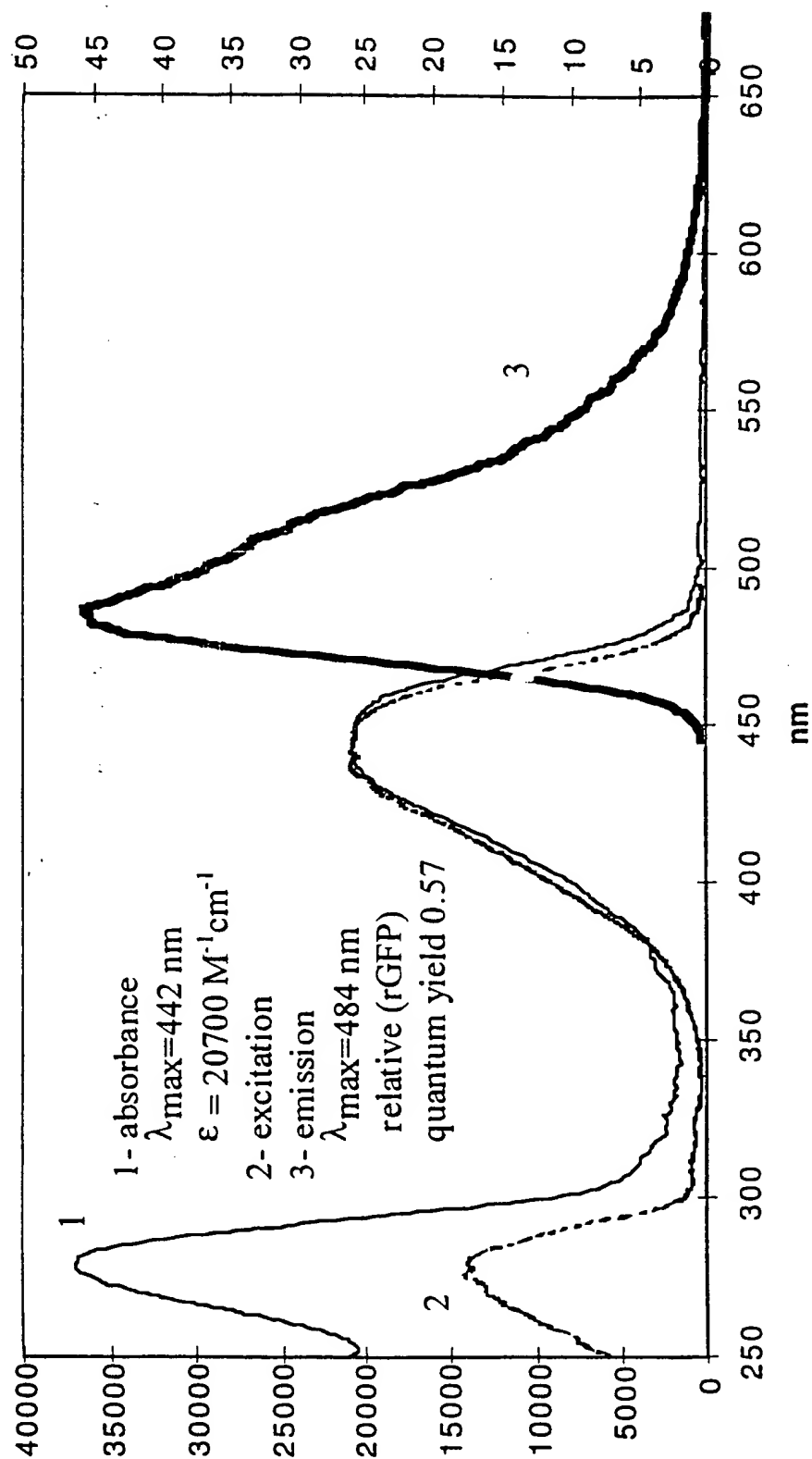


FIG. 6

dsFP484**FIG. 7**

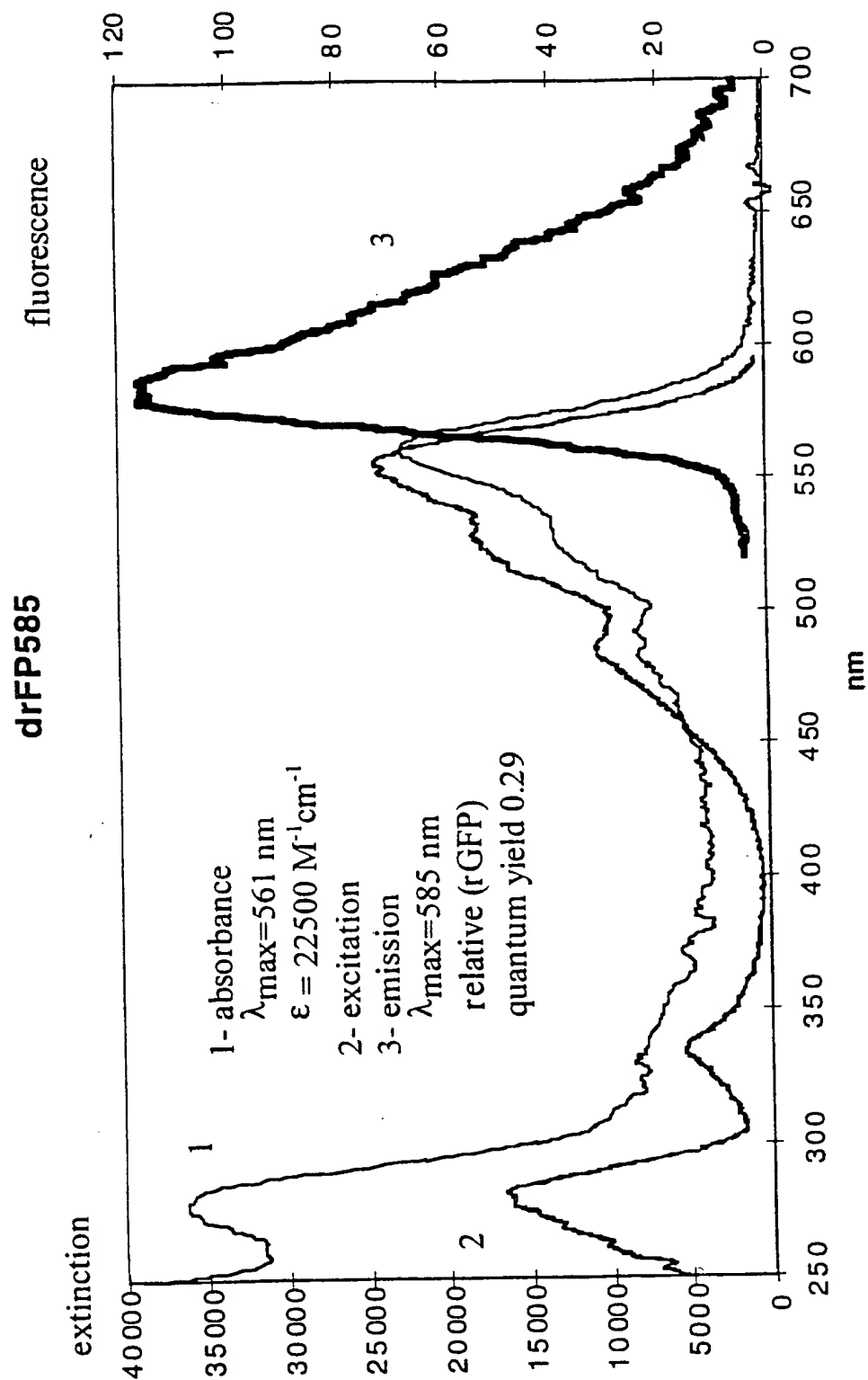


FIG. 8

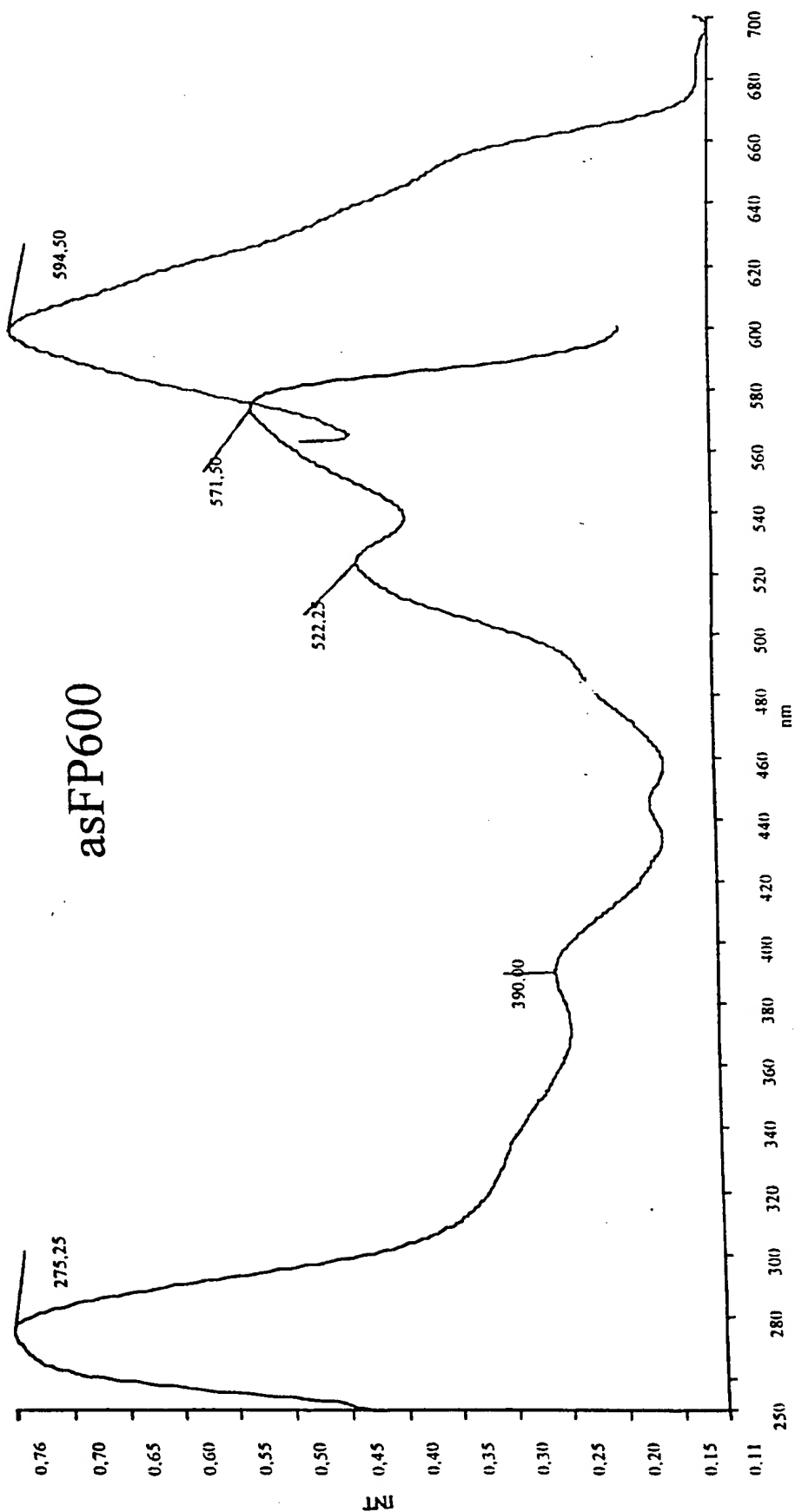


FIG. 9

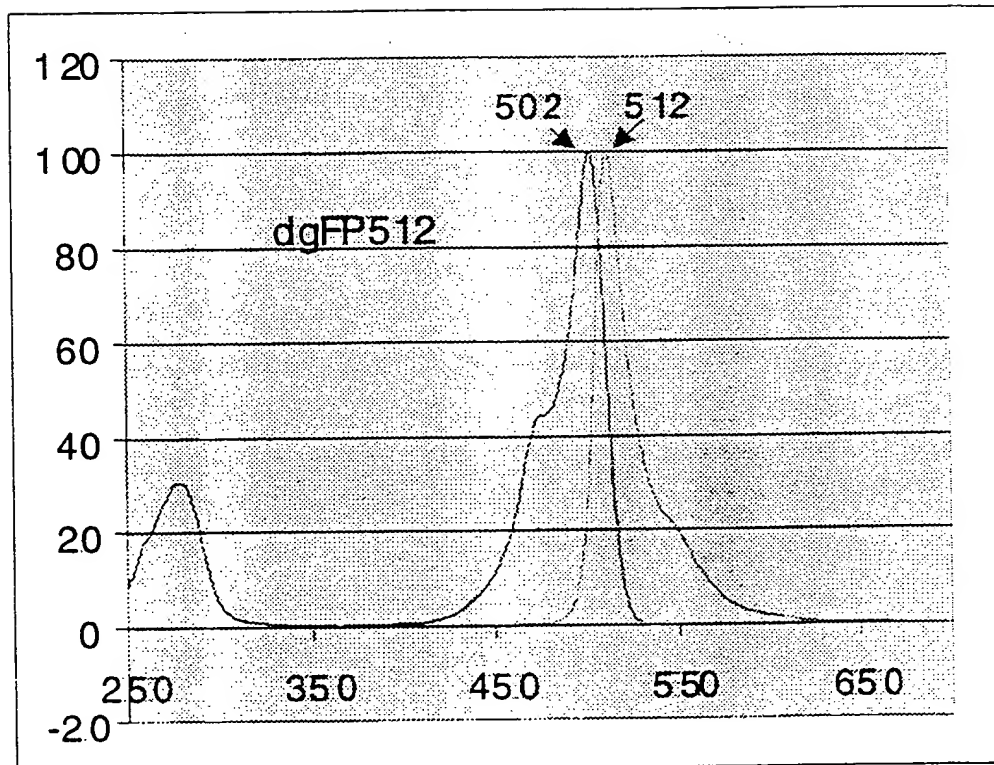


Fig. 10

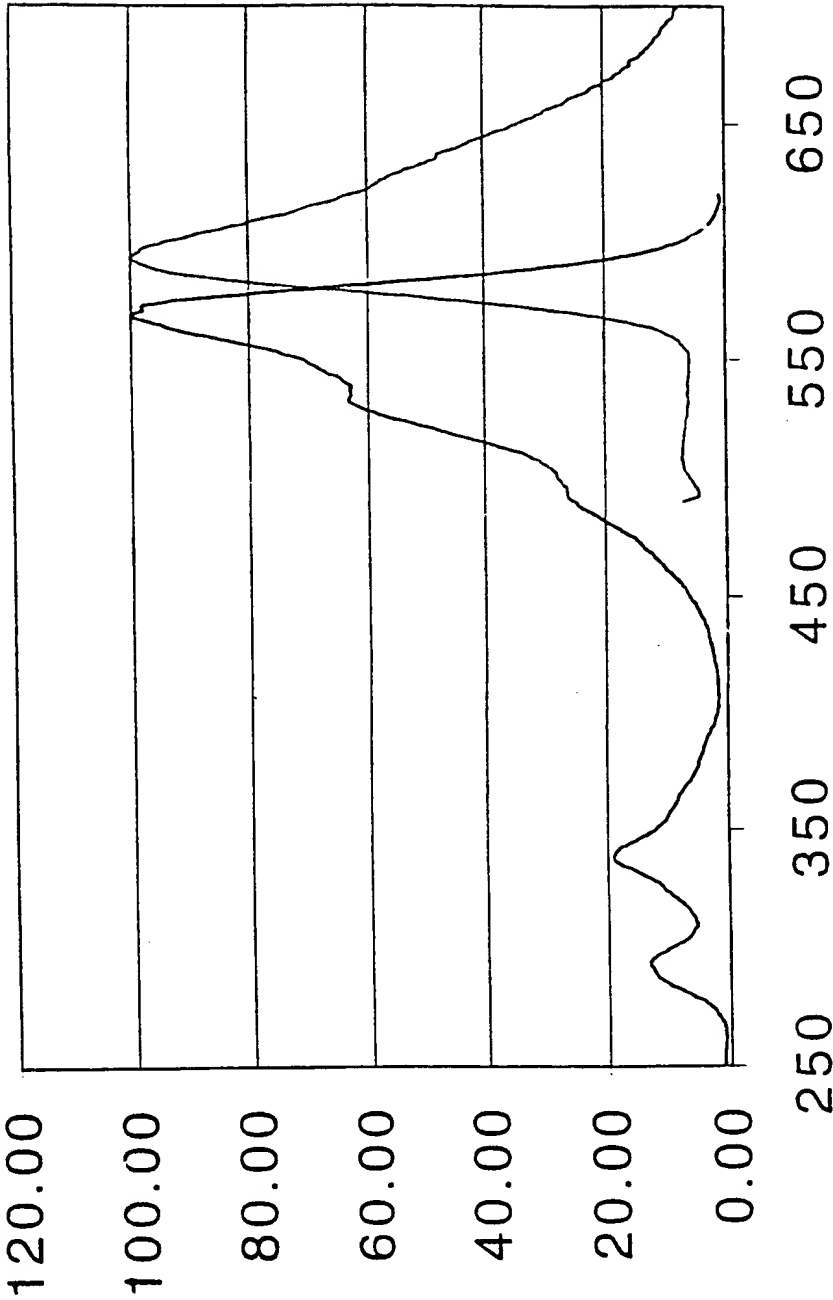


FIG. 11

SEQUENCE LISTING

<110> Lukyanov, Sergey A.
 Labas, Yulii A.
 Matz, Mikhail V.
 5 Fradkov, Arcady F.
 <120> Fluorescent proteins from non-bioluminescent
 species of Class Anthozoa, genes encoding such
 proteins and uses thereof
 <130> D6196PCT
 10 <141> 1999-12-10
 <150> 09/210,330
 <151> 1998-12-11
 <160> 63
 15 <210> 1
 <211> 25
 <212> DNA
 <213> artificial sequence
 <220>
 20 <221> primer_bind
 <223> primer TN3 used in cDNA synthesis and RACE
 <400> 1
 cgccagtcgac cggtttttttt ttttt 25
 25 <210> 2
 <211> 23
 <212> DNA
 <213> artificial sequence
 <220>
 30 <221> primer_bind
 <223> primer TS used in cDNA synthesis and RACE
 <400> 2
 aagcagtgggt atcaacgcag agt 23
 35 <210> 3
 <211> 6
 <212> PRT

SEQ 1/28

<213> *Aequorea victoria*
 <220>
 <222> 21
 <223> amino acid sequence of a key stretch on which
 5 primer NGH is based; Xaa at position 21
 represents unknown
 <400> 3
 Gly Xaa Val Asn Gly His
 5
 10
 <210> 4
 <211> 20
 <212> DNA
 <213> artificial sequence
 15
 <220>
 <221> primer_bind
 <222> 12
 <223> primer NGH used for isolation of fluorescent
 20 protein; n at position 12 represents any of the
 four bases
 <400> 4
 gayggctgcg tnaayggdca 20
 <210> 5
 25 <211> 5
 <212> PRT
 <213> *Aequorea victoria*
 <220>
 <222> 31..35
 30 <223> amino acid sequence of a key stretch on which
 primers GEGa and GEGb are based
 <400> 5
 Gly Glu Gly Glu Gly
 5
 35
 <210> 6
 <211> 20

SEQ 2/28

<212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 5 <223> primer GEGa used for isolation of fluorescent
 protein
 <400> 6

gttacaggtg arggmgargg

20

10 <210> 7
 <211> 20
 <212> DNA
 <213> artificial sequence
 <220>
 15 <221> primer_bind
 <223> primer GEGb used for isolation of fluorescent
 protein
 <400> 7

gttacaggtg arggkgargg

20

20
 <210> 8
 <211> 5
 <212> PRT
 <213> *Aequorea victoria*
 25 <220>
 <222> 31...35
 <223> amino acid sequence of a key stretch on which
 primers GNGa and GNGB are based
 <400> 8

30 Gly Glu Gly Asn Gly
 5

<210> 9
 <211> 20
 35 <212> DNA
 <213> artificial sequence
 <220>

SEQ 3/28

<221> primer_bind
 <223> primer GNGa used for isolation of fluorescent
 protein
 <400> 9
 5 gttacaggtg arggmaaygg 20
 <210> 10
 <211> 20
 <212> DNA
 10 <213> artificial sequence
 <220>
 <221> primer_bind
 <223> primer GNGb used for isolation of fluorescent
 protein
 15 <400> 10
 gttacaggtg arggkaaygg 20
 <210> 11
 <211> 5
 20 <212> PRT
 <213> *Aequorea victoria*
 <220>
 <222> 127..131
 <223> amino acid sequence of a key stretch on which
 25 primer NFP is based
 <400> 11
 Gly Met Asn Phe Pro
 5
 30 <210> 12
 <211> 5
 <212> PRT
 <213> *Aequorea victoria*
 <220>
 35 <222> 127...131
 <223> amino acid sequence of a key stretch on which
 primer NFP is based

SEQ 4/28

<400> 12
 Gly Val Asn Phe Pro
 5
 5 <210> 13
 <211> 20
 <212> DNA
 <213> artificial sequence
 <220>
 10 <221> primer_bind
 <223> primer NFP used for isolation of fluorescent
 protein
 <400> 13
 ttccayggtr tgaayttycc 20
 15
 <210> 14
 <211> 4
 <212> ERT
 <213> *Aequorea victoria*
 20 <220>
 <222> 134..137
 <223> amino acid sequence of a key stretch on which
 primers PVMa and PVMb are based
 <400> 14
 25 Gly Pro Val Met
 <210> 15
 <211> 21
 30 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 <222> 15
 35 <223> primer PVMa used for isolation of fluorescent
 protein; n at position 15 represents any of the

SEQ 5/28

four bases
 <400> 15
 cctgccrayg gtcnngtmat g 21

5 <210> 16
 <211> 21
 <212> DNA
 <213> artificial sequence
 <220>
 10 <221> primer_bind
 <222> 15
 <223> primer PVMb used for isolation of fluorescent
 protein; n at position 15 represents any of the
 four bases

15 <400> 16
 cctgccrayg gtcnngtkat g 21

<210> 17
 <211> 47
 20 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 <223> primer T7-TN3 used in cDNA synthesis and RACE

25 <400> 17
 gtaatacgac tcactatagg gccgcagtcg accgtttttt tttttt 47

<210> 18
 <211> 45
 30 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 <223> primer T7-TS used in cDNA synthesis and RACE

35 <400> 18
 gtaatacgac tcactatagg gcaagcagtg gtatcaacgc agagt 45

SEQ 6/28

<210> 19
 <211> 22
 <212> DNA
 5 <213> artificial sequence
 <220>
 <221> primer_bind
 <223> primer T7 used in cDNA synthesis and RACE
 <400> 19

10 gtaatacgac tcactatagg gc 22

<210> 20
 <211> 21
 <212> DNA
 15 <213> artificial sequence
 <220>
 <221> primer_bind
 <223> gene-specific primer used for 5'-RACE for
Anemonia majano
 20 <400> 20

gaaatagtca ggcatactgg t 21

<210> 21
 <211> 20
 25 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 <223> gene-specific primer used for 5'-RACE for
Anemonia majano
 30 <400> 21

gtcaggcata ctggtaggat 20

<210> 22
 35 <211> 21

SEQ 7/28

<212> DNA
<213> artificial sequence
<220>
<221> primer_bind
5 <223> gene-specific primer used for 5'-RACE for
Clavularia sp.
<400> 22

cttgaaatag tctgctatat c 21

10 <210> 23
<211> 19
<212> DNA
<213> artificial sequence
<220>
15 <221> primer_bind
<223> gene-specific primer used for 5'-RACE for
Clavularia sp.
<400> 23

tctgctatat cgtctgggt 19

20
<210> 24
<211> 23
<212> DNA
<213> artificial sequence
25 <220>
<221> primer_bind
<223> gene-specific primer used for 5'-RACE for
Zoanthus sp.
<400> 24

30 gttcttgaaa tagtctacta tgt 23

<210> 25
<211> 20
<212> DNA
35 <213> artificial sequence

SEQ 8/28

<220>
 <221> primer_bind
 <223> gene-specific primer used for 5'-RACE for
Zoanthus sp.
 5 <400> 25
 gtctactatg tcttgaggat 20

 <210> 26
 <211> 19
 10 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 <223> gene-specific primer used for 5'-RACE for
 15 *Discosoma sp.* "red"
 <400> 26
 caagcaaattg gcaaaggctc 19

 <210> 27
 20 <211> 19
 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 25 <223> gene-specific primer used for 5'-RACE for
Discosoma sp. "red"
 <400> 27
 cggtattgtg gccttcgta 19

 30 <210> 28
 <211> 19
 <212> DNA
 <213> artificial sequence
 <220>
 35 <221> primer_bind
 <223> gene-specific primer used for 5'-RACE for

SEQ 9/28

Discosoma striata

<400> 28

ttgtcttctt ctgcacaac

19

5 <210> 29

<211> 17

<212> DNA

<213> artificial sequence

<220>

10 <221> primer_bind

<223> gene-specific primer used for 5'-RACE for
Discosoma striata

<400> 29

ctgcacaacg ggtccat

17

15

<210> 30

<211> 20

<212> DNA

<213> artificial sequence

20

<220>

<221> primer_bind

<223> gene-specific primer used for 5'-RACE for
Anemonia sulcata

<400> 30

25 cctctatctt catttctgc

20

<210> 31

<211> 20

<212> DNA

30 <213> artificial sequence

<220>

<221> primer_bind

<223> gene-specific primer used for 5'-RACE for
Anemonia sulcata

35 <400> 31

tatcttcatt tctgcgtac

20

SEQ 10/28

<210> 32
 <211> 19
 <212> DNA
 5 <213> artificial sequence
 <220>
 <221> primer_bind
 <223> gene-specific primer used for 5'-RACE for
Discosoma sp. "magenta"
 10 <400> 32

ttcagcaccc catcacgag 19

<210> 33
 <211> 19
 15 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 <223> gene-specific primer used for 5'-RACE for
 20 *Discosoma sp. "magenta"*
 <400> 33

acgctcagag ctgggttcc 19

<210> 34
 25 <211> 22
 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 30 <223> gene-specific primer used for 5'-RACE for
Discosoma sp. "green"
 <400> 34

ccctcagcaa tccatcacgt tc 22

35 <210> 35
 <211> 20
 <212> DNA

SEQ 11/28

<213> artificial sequence
 <220>
 <221> primer_bind
 <223> gene-specific primer used for 5'-RACE for
 5 *Discosoma* sp. "green"
 <400> 35
 attatctcag tggatgggttc 20
 <210> 36
 10 <211> 31
 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 15 <223> upstream primer used to obtain full coding region
 of nFPs from *Anemonia majano*
 <400> 36
 acatggatcc gctctttcaa acaagtttat c 31
 20 <210> 37
 <211> 34
 <212> DNA
 <213> artificial sequence
 <220>
 25 <221> primer_bind
 <223> downstream primer used to obtain full coding
 region of nFPs from *Anemonia majano*
 <400> 37
 tagtactcga gcttattcgt atttcagtga aatc 34
 30 <210> 38
 <211> 29
 <212> DNA
 <213> artificial sequence
 35 <220>
 <221> primer_bind
 <223> upstream primer used to obtain full coding region

SEQ 12/28

of nFPs from *Clavularia sp.*

<400> 38

acatggatcc aacatttttt tgagaaacg

29

5 <210> 39

<211> 28

<212> DNA

<213> artificial sequence

<220>

10 <221> primer_bind

<223> upstream primer used to obtain full coding region
of nFPs from *Clavularia sp.*

<400> 39

acatggatcc aaagctctaa ccaccatg

28

15

<210> 40

<211> 31

<212> DNA

<213> artificial sequence

20 <220>

<221> primer_bind

<223> downstream primer used to obtain full coding
region of nFPs from *Clavularia sp.*

<400> 40

25 tagtactcga gcaacacaaa ccctcagaca a

31

<210> 41

<211> 28

<212> DNA

30 <213> artificial sequence

<220>

<221> primer_bind

<223> upstream primer used to obtain full coding region
of nFPs from *Zoanthus sp.*

35 <400> 41

acatggatcc gctcagtc aa agcagcgt

28

SEQ 13/28

5 <210> 42
 <211> 32
 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 <223> downstream primer used to obtain full coding
 region of nFPs from *Zoanthus sp.*
 10 <400> 42
 tagtactcga ggttggaact acattcttat ca 32

 15 <210> 43
 <211> 31
 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 <223> upstream primer used to obtain full coding region
 20 of nFPs from *Discosoma sp.* "red"
 <400> 43
 acatggatcc aggtcttcca agaatgttat c 31

 25 <210> 44
 <211> 29
 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 30 <223> downstream primer used to obtain full coding
 region of nFPs from *Discosoma sp.* "red"
 <400> 44
 tagtactcga ggagccaagt tcagcctta 29

 35 <210> 45
 <211> 28
 <212> DNA

SEQ 14/28

<213> artificial sequence
 <220>
 <221> primer_bind
 <223> upstream primer used to obtain full coding region
 5 of nFPs from *Discosoma striata*
 <400> 45
 acatggatcc agttggtcca agagtgtg 28
 <210> 46
 10 <211> 28
 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 15 <223> downstream primer used to obtain full coding
 region of nFPs from *Discosoma striata*
 <400> 46
 tagcgagctc tatcatgcct cgtcacct 28
 <210> 47
 20 <211> 31
 <212> DNA
 <213> artificial sequence
 <220>
 25 <221> primer_bind
 <223> upstream primer used to obtain full coding region
 of nFPs from *Anemonia sulcata*
 <400> 47
 acatggatcc gcttcctttt taaagaagac t 31
 30 <210> 48
 <211> 28
 <212> DNA
 <213> artificial sequence
 35 <220>
 <221> primer_bind
 <223> downstream primer used to obtain full coding

SEQ 15/28

region of nFPs from *Anemonia sulcata*

<400> 48

tagtactcga gtccttggga gcggcttg 28

5 <210> 49

<211> 30

<212> DNA

<213> artificial sequence

<220>

10 <221> primer_bind

<223> upstream primer used to obtain full coding region
of nFPs from *Discosoma sp. "magenta"*

<400> 49

acatggatcc agttgttcca agaatgtgat 30

15

<210> 50

<211> 26

<212> DNA

<213> artificial sequence

20 <220>

<221> primer_bind

<223> downstream primer used to obtain full coding
region of nFPs from *Discosoma sp. "magenta"*

<400> 50

25 tagtactcga ggccattacg ctaatc 26

<210> 51

<211> 31

<212> DNA

30 <213> artificial sequence

<220>

<221> primer_bind

<223> upstream primer used to obtain full coding region
of nFPs from *Discosoma sp. "green"*

35 <400> 51

acatggatcc agtgcactta aagaagaaat g 31

SEQ 16/28

<210> 52
 <211> 29
 <212> DNA
 5 <213> artificial sequence
 <220>
 <221> primer_bind
 <223> downstream primer used to obtain full coding
 region of nFPs from *Discosoma sp.* "green"
 10 <400> 52

tagtactcga gattcgggtt aatgccttg 29

<210> 53
 <211> 33
 15 <212> DNA
 <213> artificial sequence
 <220>
 <221> primer_bind
 <223> TS-oligo used in cDNA synthesis and RACE
 20 <400> 53

aagcagtggg atcaacgcag agtacgcrgr grg 33

<210> 54
 <211> 238
 25 <212> PRT
 <213> *Aequorea victoria*
 <220>
 <223> amino acid sequence of GFP
 <400> 54
 30 Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 5 10 15
 Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser
 20 25 30
 Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys
 35 35 40 45
 Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu
 50 55 60

SEQ 17/28

	Val	Thr	Thr	Phe	Ser	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro
					65					70					75
	Asp	His	Met	Lys	Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu
					80					85					90
5	Gly	Tyr	Val	Gln	Glu	Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn
					95					100					105
	Tyr	Lys	Thr	Arg	Ala	Glu	Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val
					110					115					120
	Asn	Arg	Ile	Glu	Leu	Lys	Gly	Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn
10					125					130					135
	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	Asn	Tyr	Asn	Ser	His	Asn	Val
					140					145					150
	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	Gly	Ile	Lys	Val	Asn	Phe
					155					160					165
15	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	Val	Gln	Leu	Ala	Asp
					170					175					180
	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly	Pro	Val	Leu	Leu
					185					190					195
	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu	Ser	Lys	Asp
20					200					205					210
	Pro	Asn	Glu	Lys	Arg	Asp	His	Met	Val	Leu	Leu	Glu	Phe	Val	Thr
					215					220					225
	Ala	Ala	Gly	Ile	Thr	His	Gly	Met	Asp	Glu	Leu	Tyr	Lys		
					230					235					
25															
	<210>				55										
	<211>				229										
	<212>				PRT										
	<213>				<i>Anemonia majano</i>										
30	<220>														
	<223>				amino acid sequence of amFP486										
	<400>				55										
	Met	Ala	Leu	Ser	Asn	Lys	Phe	Ile	Gly	Asp	Asp	Met	Lys	Met	Thr
					5					10					15
35	Tyr	His	Met	Asp	Gly	Cys	Val	Asn	Gly	His	Tyr	Phe	Thr	Val	Lys
					20					25					30
	Gly	Glu	Gly	Asn	Gly	Lys	Pro	Tyr	Glu	Gly	Thr	Gln	Thr	Ser	Thr
					35					40					45

SEQ 18/28

Phe Lys Val Thr Met Ala Asn Gly Gly Pro Leu Ala Phe Ser Phe
 50 55 60
 Asp Ile Leu Ser Thr Val Phe Lys Tyr Gly Asn Arg Cys Phe Thr
 65 70 75
 5 Ala Tyr Pro Thr Ser Met Pro Asp Tyr Phe Lys Gln Ala Phe Pro
 80 85 90
 Asp Gly Met Ser Tyr Glu Arg Thr Phe Thr Tyr Glu Asp Gly Gly
 95 100 105
 Val Ala Thr Ala Ser Trp Glu Ile Ser Leu Lys Gly Asn Cys Phe
 10 110 115 120
 Glu His Lys Ser Thr Phe His Gly Val Asn Phe Pro Ala Asp Gly
 125 130 135
 Pro Val Met Ala Lys Lys Thr Thr Gly Trp Asp Pro Ser Phe Glu
 140 145 150
 15 Lys Met Thr Val Cys Asp Gly Ile Leu Lys Gly Asp Val Thr Ala
 155 160 165
 Phe Leu Met Leu Gln Gly Gly Gly Asn Tyr Arg Cys Gln Phe His
 170 175 180
 Thr Ser Tyr Lys Thr Lys Lys Pro Val Thr Met Pro Pro Asn His
 20 185 190 195
 Val Val Glu His Arg Ile Ala Arg Thr Asp Leu Asp Lys Gly Gly
 200 205 210
 Asn Ser Val Gln Leu Thr Glu His Ala Val Ala His Ile Thr Ser
 215 220 225
 25 Val Val Pro Phe

<210> 56

<211> 266

30 <212> PRT

<213> *Clavularia* sp.

<220>

<223> amino acid sequence of cFP484

<400> 56

35 Met Lys Cys Lys Phe Val Phe Cys Leu Ser Phe Leu Val Leu Ala
 5 10 15
 Ile Thr Asn Ala Asn Ile Phe Leu Arg Asn Glu Ala Asp Phe Glu

	20	25	30
	Glu Lys Thr Phe Arg Ile Pro Lys Ala Leu Thr Thr Met Gly Val		
	35	40	45
	Ile Lys Pro Asp Met Lys Ile Lys Leu Lys Met Glu Gly Asn Val		
5	50	55	60
	Asn Gly His Ala Phe Val Ile Glu Gly Glu Gly Glu Gly Lys Pro		
	65	70	75
	Tyr Asp Gly Thr His Thr Leu Asn Leu Glu Val Lys Glu Gly Ala		
	80	85	90
10	Pro Leu Pro Phe Ser Tyr Asp Ile Leu Ser Asn Ala Phe Gln Tyr		
	95	100	105
	Gly Asn Arg Ala Leu Thr Lys Tyr Pro Asp Asp Ile Ala Asp Tyr		
	110	115	120
	Phe Lys Gln Ser Phe Pro Glu Gly Tyr Ser Trp Glu Arg Thr Met		
15	125	130	135
	Thr Phe Glu Asp Lys Gly Ile Val Lys Val Lys Ser Asp Ile Ser		
	140	145	150
	Met Glu Glu Asp Ser Phe Ile Tyr Glu Ile Arg Phe Asp Gly Met		
	155	160	165
20	Asp Phe Pro Pro Asn Gly Pro Val Met Gln Lys Lys Thr Leu Lys		
	170	175	180
	Trp Glu Pro Ser Thr Glu Ile Met Tyr Val Arg Asp Gly Val Leu		
	185	190	195
	Val Gly Asp Ile Ser His Ser Leu Leu Leu Glu Gly Gly Gly His		
25	200	205	210
	Tyr Arg Cys Asp Phe Lys Ser Ile Tyr Lys Ala Lys Lys Val Val		
	215	220	225
	Lys Leu Pro Asp Tyr His Phe Val Asp His Arg Ile Glu Ile Leu		
	230	235	240
30	Asn His Asp Lys Asp Tyr Asn Lys Val Thr Leu Tyr Glu Asn Ala		
	245	250	255
	Val Ala Arg Tyr Ser Leu Leu Pro Ser Gln Ala		
	260	265	
35	<210>	57	
	<211>	230	
	<212>	PRT	

SEQ 20/28

<213> *Zoanthus sp.*

<220>

<223> amino acid sequence of zFP506

<400> 57

```

5  Ala Gln Ser Lys His Gly Leu Thr Lys Glu Met Thr Met Lys Tyr
      5                      10                      15
    Arg Met Glu Gly Cys Val Asp Gly His Lys Phe Val Ile Thr Gly
      20                      25                      30
    Glu Gly Ile Gly Tyr Pro Phe Lys Gly Lys Gln Ala Ile Asn Leu
10      35                      40                      45
    Cys Val Val Glu Gly Gly Pro Leu Pro Phe Ala Glu Asp Ile Leu
      50                      55                      60
    Ser Ala Ala Phe Asn Tyr Gly Asn Arg Val Phe Thr Glu Tyr Pro
      65                      70                      75
15  Gln Asp Ile Val Asp Tyr Phe Lys Asn Ser Cys Pro Ala Gly Tyr
      80                      85                      90
    Thr Trp Asp Arg Ser Phe Leu Phe Glu Asp Gly Ala Val Cys Ile
      95                      100                     105
    Cys Asn Ala Asp Ile Thr Val Ser Val Glu Glu Asn Cys Met Tyr
20      110                     115                     120
    His Glu Ser Lys Phe Tyr Gly Val Asn Phe Pro Ala Asp Gly Pro
      125                     130                     135
    Val Met Lys Lys Met Thr Asp Asn Trp Glu Pro Ser Cys Glu Lys
      140                     145                     150
25  Ile Ile Pro Val Pro Lys Gln Gly Ile Leu Lys Gly Asp Val Ser
      155                     160                     165
    Met Tyr Leu Leu Leu Lys Asp Gly Gly Arg Leu Arg Cys Gln Phe
      170                     175                     180
    Asp Thr Val Tyr Lys Ala Lys Ser Val Pro Arg Lys Met Pro Asp
30      185                     190                     195
    Trp His Phe Ile Gln His Lys Leu Thr Arg Glu Asp Arg Ser Asp
      200                     205                     210
    Ala Lys Asn Gln Lys Trp His Leu Thr Glu His Ala Ile Ala Ser
      215                     220                     225
35  Gly Ser Ala Leu Pro
      230

```

SEQ 21/28

<210> 58
 <211> 230
 <212> PRT
 5 <213> *Zoanthus sp.*
 <220>
 <223> amino acid sequence of zFP538
 <400> 58

	Met	Ala	His	Ser	Lys	His	Gly	Leu	Lys	Glu	Glu	Met	Thr	Met	Lys
10					5					10					15
	Tyr	His	Met	Glu	Gly	Cys	Val	Asn	Gly	His	Lys	Phe	Val	Ile	Thr
					20					25					30
	Gly	Glu	Gly	Ile	Gly	Tyr	Pro	Phe	Lys	Gly	Lys	Gln	Thr	Ile	Asn
					35					40					45
15	Leu	Cys	Val	Ile	Glu	Gly	Gly	Pro	Leu	Pro	Phe	Ser	Glu	Asp	Ile
					50					55					60
	Leu	Ser	Ala	Gly	Phe	Lys	Tyr	Gly	Asp	Arg	Ile	Phe	Thr	Glu	Tyr
					65					70					75
	Pro	Gln	Asp	Ile	Val	Asp	Tyr	Phe	Lys	Asn	Ser	Cys	Pro	Ala	Gly
20					80					85					90
	Tyr	Thr	Trp	Gly	Ser	Phe	Leu	Phe	Glu	Asp	Gly	Ala	Val	Cys	Ile
					95					100					105
	Cys	Asn	Val	Asp	Ile	Thr	Val	Ser	Val	Lys	Glu	Asn	Cys	Ile	Tyr
					110					115					120
25	His	Lys	Ser	Ile	Phe	Asn	Gly	Met	Asn	Phe	Pro	Ala	Asp	Gly	Pro
					125					130					135
	Val	Met	Lys	Lys	Met	Thr	Thr	Asn	Trp	Glu	Ala	Ser	Cys	Glu	Lys
					140					145					150
	Ile	Met	Pro	Val	Pro	Lys	Gln	Gly	Ile	Leu	Lys	Gly	Asp	Val	Ser
30					155					160					165
	Met	Tyr	Leu	Leu	Leu	Lys	Asp	Gly	Gly	Arg	Tyr	Arg	Cys	Gln	Phe
					170					175					180
	Asp	Thr	Val	Tyr	Lys	Ala	Lys	Ser	Val	Pro	Ser	Lys	Met	Pro	Glu
					185					190					195
35	Trp	His	Phe	Ile	Gln	His	Lys	Leu	Leu	Arg	Glu	Asp	Arg	Ser	Asp
					200					205					210
	Ala	Lys	Asn	Gln	Lys	Trp	Gln	Leu	Thr	Glu	His	Ala	Ile	Ala	Phe
					215					220					225

SEQ 22/28

Pro Ser Ala Leu Ala

230

5 <210> 59
 <211> 232
 <212> PRT
 <213> *Discosoma striata*
 <220>
 10 <223> amino acid sequence of dsFP483
 <400> 59

	Met	Ser	Cys	Ser	Lys	Ser	Val	Ile	Lys	Glu	Glu	Met	Leu	Ile	Asp
					5					10					15
	Leu	His	Leu	Glu	Gly	Thr	Phe	Asn	Gly	His	Tyr	Phe	Glu	Ile	Lys
15					20					25					30
	Gly	Lys	Gly	Lys	Gly	Gln	Pro	Asn	Glu	Gly	Thr	Asn	Thr	Val	Thr
					35					40					45
	Leu	Glu	Val	Thr	Lys	Gly	Gly	Pro	Leu	Pro	Phe	Gly	Trp	His	Ile
					50					55					60
20	Leu	Cys	Pro	Gln	Phe	Gln	Tyr	Gly	Asn	Lys	Ala	Phe	Val	His	His
					65					70					75
	Pro	Asp	Asn	Ile	His	Asp	Tyr	Leu	Lys	Leu	Ser	Phe	Pro	Glu	Gly
					80					85					90
	Tyr	Thr	Trp	Glu	Arg	Ser	Met	His	Phe	Glu	Asp	Gly	Gly	Leu	Cys
25					95					100					105
	Cys	Ile	Thr	Asn	Asp	Ile	Ser	Leu	Thr	Gly	Asn	Cys	Phe	Tyr	Tyr
					110					115					120
	Asp	Ile	Lys	Phe	Thr	Gly	Leu	Asn	Phe	Pro	Pro	Asn	Gly	Pro	Val
					125					130					135
30	Val	Gln	Lys	Lys	Thr	Thr	Gly	Trp	Glu	Pro	Ser	Thr	Glu	Arg	Leu
					140					145					150
	Tyr	Pro	Arg	Asp	Gly	Val	Leu	Ile	Gly	Asp	Ile	His	His	Ala	Leu
					155					160					165
	Thr	Val	Glu	Gly	Gly	Gly	His	Tyr	Ala	Cys	Asp	Ile	Lys	Thr	Val
35					170					175					180
	Tyr	Arg	Ala	Lys	Lys	Ala	Ala	Leu	Lys	Met	Pro	Gly	Tyr	His	Tyr
					185					190					195

SEQ 23/28

	Val	Asp	Thr	Lys	Leu	Val	Ile	Trp	Asn	Asn	Asp	Lys	Glu	Phe	Met
					200					205					210
	Lys	Val	Glu	Glu	His	Glu	Ile	Ala	Val	Ala	Arg	His	His	Pro	Phe
					215					220					225
5	Tyr	Glu	Pro	Lys	Lys	Asp	Lys								
					230										
	<210>				60										
	<211>				225										
10	<212>				PRT										
	<213>				<i>Discosoma</i>										
	<220>														
	<223>				amino acid sequence of drFP583										
	<400>				60										
15	Met	Arg	Ser	Ser	Lys	Asn	Val	Ile	Lys	Glu	Phe	Met	Arg	Phe	Lys
					5					10					15
	Val	Arg	Met	Glu	Gly	Thr	Val	Asn	Gly	His	Glu	Phe	Glu	Ile	Glu
					20					25					30
	Gly	Glu	Gly	Glu	Gly	Arg	Pro	Tyr	Glu	Gly	His	Asn	Thr	Val	Lys
20					35					40					45
	Leu	Lys	Val	Thr	Lys	Gly	Gly	Pro	Leu	Pro	Phe	Ala	Trp	Asp	Ile
					50					55					60
	Leu	Ser	Pro	Gln	Phe	Gln	Tyr	Gly	Ser	Lys	Val	Tyr	Val	Lys	His
					65					70					75
25	Pro	Ala	Asp	Ile	Pro	Asp	Tyr	Lys	Lys	Leu	Ser	Phe	Pro	Glu	Gly
					80					85					90
	Phe	Lys	Trp	Glu	Arg	Val	Met	Asn	Phe	Glu	Asp	Gly	Gly	Val	Val
					95					100					105
	Thr	Val	Thr	Gln	Asp	Ser	Ser	Leu	Gln	Asp	Gly	Cys	Phe	Ile	Tyr
30					110					115					120
	Lys	Val	Lys	Phe	Ile	Gly	Val	Asn	Phe	Pro	Ser	Asp	Gly	Pro	Val
					125					130					135
	Met	Gln	Lys	Lys	Thr	Met	Gly	Trp	Glu	Ala	Ser	Thr	Glu	Arg	Leu
					140					145					150
35	Tyr	Pro	Arg	Asp	Gly	Val	Leu	Lys	Gly	Glu	Ile	His	Lys	Ala	Leu
					155					160					165
	Lys	Leu	Lys	Asp	Gly	Gly	His	Tyr	Leu	Val	Glu	Phe	Lys	Ser	Ile
					170					175					180

SEQ 24/28

	Tyr	Met	Ala	Lys	Lys	Pro	Val	Gln	Leu	Pro	Gly	Tyr	Tyr	Tyr	Val
					185					190					195
	Asp	Ser	Lys	Leu	Asp	Ile	Thr	Ser	His	Asn	Glu	Asp	Tyr	Thr	Ile
					200					205					210
5	Val	Glu	Gln	Tyr	Glu	Arg	Thr	Glu	Gly	Arg	His	His	Leu	Phe	Leu
					215					220					225
	<210>				61										
	<211>				232										
10	<212>				PRT										
	<213>				<i>Anemonia sulcata</i>										
	<220>														
	<223>				amino acid sequence of asFP600										
	<400>				61										
15	Met	Ala	Ser	Phe	Leu	Lys	Lys	Thr	Met	Pro	Phe	Lys	Thr	Thr	Ile
					5					10					15
	Glu	Gly	Thr	Val	Asn	Gly	His	Tyr	Phe	Lys	Cys	Thr	Gly	Lys	Gly
					20					25					30
	Glu	Gly	Asn	Pro	Phe	Glu	Gly	Thr	Gln	Glu	Met	Lys	Ile	Glu	Val
20					35					40					45
	Ile	Glu	Gly	Gly	Pro	Leu	Pro	Phe	Ala	Phe	His	Ile	Leu	Ser	Thr
					50					55					60
	Ser	Cys	Met	Tyr	Gly	Ser	Lys	Thr	Phe	Ile	Lys	Tyr	Val	Ser	Gly
					65					70					75
25	Ile	Pro	Asp	Tyr	Phe	Lys	Gln	Ser	Phe	Pro	Glu	Gly	Phe	Thr	Trp
					80					85					90
	Glu	Arg	Thr	Thr	Thr	Tyr	Glu	Asp	Gly	Gly	Phe	Leu	Thr	Ala	His
					95					100					105
	Gln	Asp	Thr	Ser	Leu	Asp	Gly	Asp	Cys	Leu	Val	Tyr	Lys	Val	Lys
30					110					115					120
	Ile	Leu	Gly	Asn	Asn	Phe	Pro	Ala	Asp	Gly	Pro	Val	Met	Gln	Asn
					125					130					135
	Lys	Ala	Gly	Arg	Trp	Glu	Pro	Ala	Thr	Glu	Ile	Val	Tyr	Glu	Val
					140					145					150
35	Asp	Gly	Val	Leu	Arg	Gly	Gln	Ser	Leu	Met	Ala	Leu	Lys	Cys	Pro
					155					160					165
	Gly	Gly	Arg	His	Leu	Thr	Cys	His	Leu	His	Thr	Thr	Tyr	Arg	Ser
					170					175					180

SEQ 25/28

Lys Lys Pro Ala Ser Ala Leu Lys Met Pro Gly Phe His Phe Glu
 185 190 195
 Asp His Arg Ile Glu Ile Met Glu Glu Val Glu Lys Gly Lys Cys
 200 205 210
 5 Tyr Lys Gln Tyr Glu Ala Ala Val Gly Arg Tyr Cys Asp Ala Ala
 215 220 225
 Pro Ser Lys Leu Gly His Asn
 230

10 <210> 62
 <211> 231
 <212> PRT
 <213> *Discosoma* sp. "green"
 <220>
 15 <223> amino acid sequence of dgFP512
 <400> 62

Met Ser Ala Leu Lys Glu Glu Met Lys Ile Asn Leu Thr Met Glu
 5 10 15
 Gly Val Val Asn Gly Leu Pro Phe Lys Ile Arg Gly Asp Gly Lys
 20 20 25 30
 Gly Lys Pro Tyr Gln Gly Ser Gln Glu Leu Thr Leu Thr Val Val
 35 40 45
 Lys Gly Gly Pro Leu Pro Phe Ser Tyr Asp Ile Leu Thr Thr Met
 50 55 60
 25 Phe Gln Tyr Gly Asn Arg Ala Phe Val Asn Tyr Pro Glu Asp Ile
 65 70 75
 Pro Asp Ile Phe Lys Gln Thr Cys Ser Gly Pro Asn Gly Gly Tyr
 80 85 90
 Ser Trp Gln Arg Thr Met Thr Tyr Glu Asp Gly Gly Val Cys Thr
 30 95 100 105
 Ala Thr Ser Asn Ile Ser Val Val Gly Asp Thr Phe Asn Tyr Asp
 110 115 120

SEQ 26/28

	Ile His Phe Met Gly Ala Asn Phe Pro Leu Asp Gly Pro Val Met		
	125	130	135
	Gln Lys Arg Thr Met Lys Trp Glu Pro Ser Thr Glu Ile Met Phe		
	140	145	150
5	Glu Arg Asp Gly Met Leu Arg Gly Asp Ile Ala Met Ser Leu Leu		
	155	160	165
	Leu Lys Gly Gly Gly His Tyr Arg Cys Asp Phe Glu Thr Ile Tyr		
	170	175	180
	Lys Pro Asn Lys Val Val Lys Met Pro Asp Tyr His Phe Val Asp		
10	185	190	195
	His Cys Ile Glu Ile Thr Ser Gln Gln Asp Tyr Tyr Asn Val Val		
	200	205	210
	Glu Leu Thr Glu Val Ala Glu Ala Arg Tyr Ser Ser Leu Glu Lys		
	215	220	225
15	Ile Gly Lys Ser Lys Ala		
	230		
	<210>	63	
	<211>	235	
20	<212>	PRT	
	<213>	<i>Discosoma</i> sp. "magenta"	
	<220>		
	<223>	amino acid sequence of dmFP592	
	<400>	63	
25	Met Ser Cys Ser Lys Asn Val Ile Lys Glu Phe Met Arg Phe Lys		
	5	10	15
	Val Arg Met Glu Gly Thr Val Asn Gly His Glu Phe Glu Ile Lys		
	20	25	30
	Gly Glu Gly Glu Gly Arg Pro Tyr Glu Gly His Cys Ser Val Lys		
30	35	40	45

SEQ 27/28

	Leu Met Val Thr Lys Gly Gly Pro Leu Pro Phe Ala Phe Asp Ile		
	50	55	60
	Leu Ser Pro Gln Phe Gln Tyr Gly Ser Lys Val Tyr Val Lys His		
	65	70	75
5	Pro Ala Asp Ile Pro Asp Tyr Lys Lys Leu Ser Phe Pro Glu Gly		
	80	85	90
	Phe Lys Trp Glu Arg Val Met Asn Phe Glu Asp Gly Gly Val Val		
	100	105	110
	Thr Val Ser Gln Asp Ser Ser Leu Lys Asp Gly Cys Phe Ile Tyr		
10	115	120	125
	Glu Val Lys Phe Ile Gly Val Asn Phe Pro Ser Asp Gly Pro Val		
	130	135	140
	Met Gln Arg Arg Thr Arg Gly Trp Glu Ala Ser Ser Glu Arg Leu		
	145	150	155
15	Tyr Pro Arg Asp Gly Val Leu Lys Gly Asp Ile His Met Ala Leu		
	160	165	170
	Arg Leu Glu Gly Gly Gly His Tyr Leu Val Glu Phe Lys Ser Ile		
	175	180	185
	Tyr Met Val Lys Lys Pro Ser Val Gln Leu Pro Gly Tyr Tyr Tyr		
20	190	195	200
	Val Asp Ser Lys Leu Asp Met Thr Ser His Asn Glu Asp Tyr Thr		
	205	210	215
	Val Val Glu Gln Tyr Glu Lys Thr Gln Gly Arg His His Pro Phe		
	220	225	230
25	Ile Lys Pro Leu Gln		
	235		

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/29405

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/68; C07K 14/435

US CL :435/6, 69.1; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 69.1, 968; 530/350; 424/9.6, 436/172

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
***	The sequence diskette submitted with the description was defective; thus the references listed below were obtained solely by a WORD search, and not by a search of the SEQ ID NOs.	***
X, P	MATZ et al. Fluorescent proteins from nonbioluminescent Anthozoa species. Nature Biotechnology. October 1999, Volume 17, No. 10, pages 969-973, entire document.	1-10
X, P	DE 197 18 640 A1 (WIEDENMANN) 22 July 1999, entire document.	3-10

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

18 FEBRUARY 2000

Date of mailing of the international search report

02 MAR 2000

 Name and mailing address of the ISA/US
 Commissioner of Patents and Trademarks
 Box PCT
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

GABRIELE ELISABETH BOGATSKY

Telephone No. (703) 308-0196

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☒ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☒ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.